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(57) Abstract

A gel-like liposome composition and method of preparing the same. The composition is composed of charged liposomes, at a relatively low lipid concentration, in a low-conductivity medium. The composition preferably contains a zwitterionic species at its isoelectric point. The liposomes can be designed for cosmetic use, transdermal drug delivery, or enhanced retention on mucosal tissues, such as for ophthalmic use. In one embodiment, the gel composition contains encapsulated and/or surface bound epidermal growth factor, for use particularly in surgical wound healing.

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LIPOSOME GEL COMPOSITION AND METHOD

1. Field of the Invention

The present invention relates to a high-viscosity liposome gel composition, and to methods of making and using the composition.

2. References

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3. Background of the Invention

Lipid bilayer vesicles, or liposomes, have been proposed for use in a variety of topical applications. In the cosmetics industry, liposome formulations are currently sold as a lipid supplement to enhance dry or aging skin. Liposomes may also be useful for applying compounds, such as ultraviolet-blocking agents, vitamin A, retin, and the like to the skin, to achieve greater drug solubility or skin compatibility, reduced irritation from the drug, and/or extended drug release.

Liposomes also offer the potential of improved transdermal drug delivery. It is known, for example, that liposomes are able to facilitate the uptake of

certain lipophilic compounds, such as anti-inflammatory steroid compounds, across the skin barrier (Mezei, 1982, 1983), and the drug-uptake characteristics of the liposomes can be modulated by varying lipid composition in the liposomes. Additionally, the liposomes can be formulated with coentrapped agents, such as azacycloalkane-2-ones, which facilitate transdermal uptake of drugs (U.S. Patent No. 4,316,893), to improve and/or modulate transdermal drug release characteristics.

Liposomes are also promising drug-delivery vehicles for sustained drug release on mucosal surfaces, including corneal tissue. In ophthalmic use, for example, liposomes can provide delayed drug release, and greater solubility of lipid-soluble drugs, for release at the corneal surface, and liposomes alone are useful as a lipid supplement for dry eye (U.S. Patent No. 4,818,537). Additionally, liposomes can be engineered for enhanced retention on mucosal surfaces, to extend the period of effective drug delivery with each liposome application (U.S. Patent No. 4,804,539).

In all of the above topical uses of liposomes, it is generally desirable to administer the liposome preparation in a viscous form. In particular, the ideal liposome preparation is a gel which is preferably sufficiently viscous to give persistence at the site of application, especially at a wound site or mucosal tissue site. In cosmetic applications, the gel material should be clear or translucent and preferably be non-greasy to the touch.

Heretofore, viscous liposome pastes have been prepared by forming liposomes at high lipid concentrations, for example, by concentrating dilute liposome preparations. The high lipid concentrations make these formula-

ctions relatively expensive. The viscosity of the paste material may also complicate processing steps used for example, to sterilize the liposomes or remove non-entrapped drug molecules. Further, liposome paste preparations are generally greasy to the touch.

Liposome gel formulations have been produced heretofore by suspending liposomes in gel-forming colloidal materials, such as Hydrogel™, collagen, synthetic polymers, and the like. Although liposome-in-gel formulations of this type can be prepared with desired physical properties, the gel-forming matrix itself may be toxic or otherwise incompatible with the site of application.

4. Summary of the Invention

It is therefore one object of the invention to provide a high-viscosity liposome gel composition which provides many of the above-discussed desired features of a viscous liposome formulation for topical use.

It is a more specific object of the invention to provide a high-viscosity EGF/liposome gel composition which can be applied to a wound or surgical incision, for retention and sustained release of EGF at the site of application.

It is another object of the invention to provide a method for treating a wound or incision with such composition.

The invention includes, in one aspect, a high-viscosity liposome gel composition for use in topical application to the skin, in skin wounds, and on mucosal tissue. The composition includes a suspension of charged liposomes in a low-conductivity aqueous suspension medium which has a selected pH between about 5.5 and 8.5. The charged liposomes contain between about 5-50 weight

percent charged vesicle-forming lipids, and the balance of neutral vesicle-forming lipids. The concentration of lipids in the composition is between about 7-25 weight percent and preferably between about 8-12 percent.

The aqueous suspension preferably contains a zwitterionic compound, such as a neutral amino acid, whose isoelectric point is at the selected pH of between about 5.5 and 8.5.

In one general embodiment, the charged vesicle-forming lipids include negatively charged lipids phospholipid components, such as phosphatidylglycerol (PG). One preferred liposome composition includes approximately equal weight proportions of PG, phosphatidylcholine (PC), and cholesterol.

The EGF/liposome composition of the invention includes a high-viscosity suspension of negatively charged EGF/liposomes, i.e., liposomes containing EGF in liposome-entrapped form. The EGF/liposomes contain neutral phospholipid, and at least about 10 weight percent negatively charged phospholipid, and preferably, between 20-50 weight percent each of neutral phospholipid, negatively charged phospholipid, and cholesterol. The total lipid concentration of the EGF/liposomes in the composition is at least 50 mg/g composition and preferably between 50-200 mg/g composition. The EGF may be entrapped in the EGF/liposomes by encapsulation or surface adsorption or a combination of both.

In another general embodiment, for use in administering a drug to mucosal tissue, the charged vesicle-forming lipids include positively charged lipid components, such as a phosphatidylethanolamine conjugate prepared by derivatizing phosphatidylethanolamine with a basic amino

acid, or a benzylamine lipid, such as benzyldimethyl-stearylammonium chloride (BDSA).

The liposome gel composition is formed, according to the method of the invention, by adding a mixture of vesicle-forming lipids containing between about 10-50 weight percent components having a common charge at a selected pH between about 5.5 and 8.5, with a low-conductivity aqueous suspension medium, at a final total lipid concentration of between about 7-25 weight percent. The lipids may be added directly to a low-conductivity aqueous medium or, alternatively, to an aqueous medium containing a zwitterionic compound whose isoelectric point is substantially different from that of the pH of the medium, such that the medium is not characterized by low conductivity. Following formation of a fluidic liposome suspension, the medium is titrated to a pH at which the zwitterionic compound is at its isoelectric point, yielding a low-conductivity condition which produces gel formation in the suspension. The liposome suspension may be more easily sized, freed of non-liposome-bound drug, filter-sterilized or otherwise processed in the more fluidic state prior to gelling.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 is a plot of viscosity of a liposome suspension formed in accordance with the present invention, as a function of concentration of univalent electrolyte;

Figures 2A-2C show the electrical potential seen by charged particles as a function of distance from the

surface of a negatively charged liposome for high lipid concentration and high inner strength (2A), low lipid concentration and high inner strength (2B), and low lipid concentration and low inner strength (2C);

Figure 3 illustrates the change in calculated Debye length as a function of univalent electrolyte for charged liposomes having lower (solid line) and higher (dashed line) surface charge densities;

Figures 4 and 5 are Scatchard plots of EGF binding to EPG/EPC and EPG/EPC/cholesterol liposomes, respectively;

Figures 6 and 7 are plots of surface pressure, at an air/water interface, of aqueous EGF (Figure 6) and EPG/-EPC/cholesterol (Figure 4), respectively, as a function of EGF and liposome concentration;

Figure 8 is a plot of change in surface pressure, as a function of initial surface pressure, in the presence and absence of EGF in EPC/EPG/cholesterol liposomes (open triangles) and PC/PG liposomes (solid circles);

Figures 9-12 show the change in free EGF available in the donor compartment of a two compartment flux chamber, plotted as a function of time for free EGF (Figure 9) and for three EGF/liposome compositions (Figures 10-12);

Figures 13-16 show the retention of radiolabled EGF, plotted as a function of time, for free EGF (Figure 13), and for three EGF/liposome compositions (Figures 14-16);

Figure 17A-17C illustrate surgical steps in a corneal implant operation; and

Figures 18A-18C are diagrammatic cross-sections of the surgical region of an eye seen in Figures 17A-17C, showing in Figure 18B the introduction of an EGF/liposome formulation prepared according to the invention, and in

Figure 18C, the residual composition in the eye after an extended release period.

Detailed Description of the Invention

I. Liposome Gel Compositions

This section describes components and methods used in forming the high-viscosity liposome composition of the invention.

A. Definitions

As used herein, the terms below have the following meaning:

1. "Neutral vesicle-forming lipids" refers to any lipid or lipid mixture (i) capable of forming stable lipid bilayer vesicles in the presence of charged vesicle-forming lipids, at a selected concentration of charged lipids between 5-50 weight percent of total lipids, and (ii) having a polar head group with no net charge at a pH between about 5.5-8.5.

2. "Charged vesicle-forming lipids" refers to any amphipathic lipid (i) capable, at a selected concentration between 5-50 weight percent, of forming stable lipid bilayers in the presence of neutral vesicle-forming lipids, and (ii) having a polar head group with a net negative or positive charge at a pH between about 5.5-8.5.

3. "Negatively charged phospholipid" refers to any vesicle-forming lipid having (i) two hydrocarbon-chain moieties which are effective to produce a stable bilayer formation, and (ii) a polar head group with a net negative charge at a pH between about 5.5-8.5.

4. "Cholesterol" refers to cholesterol or any related sterol capable of combining with phospholipids to form stable lipid-bilayer vesicles.

5. "Epidermal Growth Factor" or "EGF" refers to human-EGF (h-EGF), typically recombinantly produced human EGF (rh-EGF), and to related peptides having the requisite ability to promote the growth of a variety of cells of epithelial origin in vitro.

6. "High-viscosity" or "gel" or "gel-like" refers to a viscous, relatively non-flowable gel consistency which can be applied by squeezing from a tube or syringe, but which is sufficiently non-flowable, once applied, to be retained in bolus form at a wound or incision site for at least several hours.

7. A "low-conductivity aqueous medium" refers to an aqueous medium whose conductivity is no more than that of a fully ionized univalent electrolyte whose concentration is between about 5-10 mM. Typically, the low-conductivity medium is one which reduces the Debye length of a charged liposome by no more than half its value at a concentration of fully ionized univalent electrolyte of about 1 mM.

B. Lipid Components

The liposome gel composition formed in accordance with the invention is prepared to contain between about 50-95 weight percent neutral vesicle-forming lipids, and about 5-50 weight percent charged vesicle-forming lipids which impart a net negative or net positive charge to the liposome surfaces.

Preferred neutral vesicle-forming lipids are phospholipids, such as phosphatidylcholine (PC), and cholesterol. Neutral phospholipids lipids having a

variety of acyl chain groups of varying chain length and degree of saturation are available, or may be isolated or synthesized by well-known techniques. In general, partially unsaturated phosphatidylcholine (PC), such as egg PC (EPC) or soy PC (SPC), or fully or partially hydrogenated egg PC (HEPC) or soy PC (HSPC) are readily obtained and provide suitable liposome characteristics, such as ease of extrusion and stability.

Cholesterol and related uncharged neutral analogues thereof, such as 5,6,-cholestene and cholestane, are typically present at about 20-50 weight percent. Cholesterol is known to increase the stability of liposomes and, in the case where the phospholipid components are relatively unsaturated, to increase the packing density of the lipids in the liposomal bilayers. One advantage of cholesterol, where the liposomes are applied at a wound or surgical site, is potentially reduced toxicity due to lipid exchange between the liposomes and cells at the wound or surgical site. It has been demonstrated, for example, with several cultured tumor cell lines, that liposomes containing entrapped epidermal growth factor (EGP) inhibit cellular growth in vitro, and that for at least some cell lines, this inhibition can be greatly reduced by the addition of cholesterol to EPC liposomes (Mayhew).

Preferred negatively charged vesicle-forming lipids include negatively charged phospholipids, such as the negatively charged phospholipids phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI). One preferred negatively charged phospholipid is partially or fully saturated PG, such as egg PG (EPG). Alternatively, or in addition, the charged components may

preferably include charged cholesterol derivatives such as cholesterol sulfate and cholesterol hemisuccinate.

One preferred type of positively charged vesicle-forming lipids include positively charged phospholipids, such as phoshatidylethanolamine (PE) or phospholipids which have been derivatized at their polar head groups with amines, to produce a net positive charge. Methods of producing derivatized phospholipids of this type are described in co-owned U.S. Patent No. 4,804,539. By way of example, PE can be derivatized with basic amino acids, such as lysine, to produce vesicle-forming lipids whose positive charge is separated from the phosphate groups of the lipid by a several-atom spacer.

Another general class of positively charged vesicle-forming lipids include benzyl/aliphatic-chain amines which are (a) capable of being anchored in a liposome bilayer by virtue of the aliphatic chain, and (b) carry a net positive charge at a selected pH between about 5.5 and 8.5. The aliphatic chain is at least about 12 carbons in length, and the amine is preferably a quaternary amine whose remaining (two) nitrogen linked groups are short-chain alkyl groups, such as methyl or ethyl groups, as detailed in U.S. Patent No. 4,818,537. One preferred compound is benzyldimethylstearylammonium chloride (BDSA).

Cholesterol amines form another class of positively-charged vesicle-forming lipids which are suitable for use in the invention. Cholesterol derivatives of the type Ch-O-C-Y-N and Ch-NH-Y-N, where ChOH is cholesterol, and Y is a short carbon-containing chain, have been described.

Finally, the charged lipid component may include a lipophilic drug which tends to be tightly bound to the lipid bilayer phase of the liposomes.

The lipid components forming the liposomes contain at least about 5-10 weight percent charged lipid component, and preferably between about 20-40 weight percent charged lipid. The balance of the lipids are neutral vesicle-forming lipids. The following lipid compositions, expressed in weight percent, are exemplary:

1. HEPC:EPG, 95:5;
2. EPC:EPG, 80:20;
3. EPC:EPG, 50:50;
4. EPC:EPG:cholesterol, 50:20:30;
5. EPC:EPG:cholesterol, 33:33:33;
6. EPC:cholesterol sulfate, 80:20;
7. EPC:cholesterol:cholesterol sulfate 50:30:20;
8. FSPC:PE 80:20;
9. FSPC:lysinyl PE 80:20;
10. EPC:cholesterol:cholesterol amine 60:20:20;
11. PC:BDSA 90:10; and
12. PC:BDSA 75:25.

It is noted that the total amount of neutral and charged cholesterol together is preferably no more than 50 weight percent. Further, it is understood that the liposome composition may contain a variety of other lipid components which may enhance liposome stability, viscosity, or drug release characteristics, and/or materials cost. For example, the liposomes may include α-tocopherol, or pharmaceutically acceptable analogue thereof, at a total concentration of between about 0.1 to 2 weight percent, to improve lipid stability on storage.

EGF/liposomes formed in accordance with the invention are prepared to contain between 10-90 weight percent

neutral phospholipid, and 10-50 weight percent negatively charged phospholipid, and preferably between about 20-50 weight percent each of neutral phospholipid, negatively charged phospholipid, and cholesterol.

The negatively charged phospholipid in the composition serves two important roles. First, it imparts a negative charge to the lipid bilayer membranes, providing an electrostatic interaction between the membrane and the positively-charged EGF. The adsorption of EFG to the liposomal membrane will be discussed below. Secondly, the relatively high surface charge is important in the formation of a gel-like liposome state which is characterized by a low lipid concentration and high viscosity, as described below.

The effect of cholesterol on the rate of EGF release from EGF liposomes has been examined both in vitro and in vivo, as detailed below. Briefly, cholesterol significantly increased the half-life of EGF release in vivo. Another advantage of cholesterol in the EGF/liposome composition is potentially reduced toxicity due to lipid exchange between the liposomes and cells at the wound or surgical site, as noted above.

C. Low-Conductivity Aqueous Medium

According to an important feature of the invention, it has been discovered that hydration of vesicle-forming lipids having the above composition with a low-conductivity aqueous medium produces a liposome composition which is both gel-like in consistency and viscosity, and has a relatively low lipid concentration.

More specifically, the combination of surface charge on the liposomes, due to the presence of charged lipid component(s) and the low-conductivity aqueous medium

produces a liposome composition characterized by (a) a viscous, gel-like consistency and (b) a relatively low lipid concentration, e.g., 50-250 mg/g composition and 7-25 weight percent lipid.

The aqueous medium preferably includes a zwitterionic compound whose isoelectric point (at which the compound is effectively a non-electrolyte) is at the selected pH of the medium between pH 5.5 and 8.5. Neutral amino acids, such as glycine, isoleucine alanine, proline, and valine are preferred zwitterionic compounds. The final concentration of zwitterionic compound in the buffer is typically at least about 0.5 percent by weight and preferably between about 1-5 percent by weight, and the buffer is adjusted in pH to the isoelectric point of the compound to achieve the gel state.

As will be discussed in Section D below, the aqueous medium may initially be adjusted to a pH at which the zwitterionic compound is substantially in a charged form, so that the medium has a relatively high electrolyte concentration, i.e., a relatively high conductivity. By adjusting the pH to the isoelectric point of the zwitterionic compound, typically after lipid hydration and liposome formation, the compound becomes non-electrolytic, i.e., has the desired low conductivity. It is noted, however, that the final salt concentration of the medium, after adjusting the pH to the isoelectric point of the zwitterionic compound, must not produce a significant increase in the ionic strength of the medium. This objective can be achieved, for example, by employing volatile ammonium salts, or as described below, by employing an initial low concentration of zwitterionic compound.

The zwitterionic compound is preferably present at a concentration of between about 1-3 weight percent aqueous medium, and preferably at a concentration which renders the final composition substantially isotonic. Alternatively, or in addition, the aqueous medium may includes other non-electrolyte solute compounds, such as sugars, uncharged water-soluble drugs, and the like which produce a desired osmolarity of the final gel composition.

D. Preparing the Gel Composition

The gel composition of the invention is formed by mixing the neutral and charged vesicle-forming lipids described in Section B with a low-conductivity aqueous medium, at a final lipid concentration of between about 7-25 weight percent lipid, and preferably between about 10-15 weight percent lipid.

In one general embodiment of the method, the lipids are added directly to the low-conductivity medium, such that when the selected final lipid concentration is reached, the suspension assumes a gel-like state at room temperature.

In one procedure, vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form.

This film is covered with a selected amount of the low-conductivity medium, and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multi-lamellar vesicles (MLVs) can be shifted toward smaller sizes by hydrating

the lipids under more vigorous agitation conditions. The final concentration of liposomes is at least 70 mg/g and preferably between about 100-150 mg/g composition. The hydration step is generally effective to produce a homogeneously hydrated liposome suspension, where relatively small lipid quantities are involved.

For larger lipid amounts, the hydrated suspension may contain particles of non-hydrated or partially hydrated lipids. This suspension can be converted to a homogeneous suspension by further processing, preferably by extrusion through a defined-pore size membrane, such as a 2 micron pore size polycarbonate membrane. The extrusion step, of course, also reduces the size heterogeneity in the suspension. This general procedure for preparing a liposome gel suspension is illustrated in Example 1.

In another procedure, the lipids are added to the low-conductivity medium by injecting the lipids in a lipid-in-solvent solution into the medium, until the desired lipid concentration (gel viscosity) is reached. This method is illustrated in Example 7.

It will be appreciated that water-soluble drugs or agents can be encapsulated in the liposomes formed in the gel by dissolving the drug or agent in the hydration medium. Similarly, a lipophilic compound can be conveniently added to the lipid mixture prior to hydration, for preparing liposomes with entrapped lipophilic drug.

In a second general embodiment of the method, the lipids are added to an aqueous medium containing a zwitterionic compound, at a pH which is substantially different from the isoelectric point of the compound. In particular, the concentration of zwitterionic molecules having a net positive or negative charge is such that the aqueous medium cannot be characterized by low conduc-

tivity. Typically the medium contains at least about 20 mM zwitterionic compound having a net positive or negative charge. For example, the medium may be 100 mM zwitterionic compound, at a pH at which 20 percent of the compound has a net charge.

The liposome suspension formed in the aqueous medium is relatively fluidic, or non-viscous, being characterized by high flow characteristics. Because of its low viscosity, this suspension is easily processed to achieve desired liposome/suspension characteristics. For example, the suspension may be further processed to (a) achieve smaller and or more uniform liposome sizes, (b) remove free water-soluble drug and/or (c) sterile the EGF/liposome preparation.

A variety of techniques are available for reducing liposomes to a desired size range, including sonication, homogenization and extrusion through a defined-pore size membrane. Extrusion of liposome through a small-pore polycarbonate membrane has been used successfully, as has extrusion through asymmetric ceramic membranes (U.S. Patent No. 4,737,323). The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Free water-soluble drug can be removed, if desired, by conventional centrifugation, ultrafiltration, or gel filtration (molecular sieve) methods. When the liposomes are sized by extrusion, free drug is preferably removed following the extrusion step.

The liposome suspension may be sterilized, after sizing, by filtration through a conventional depth filter, typically having a 0.22 micron particle exclusion size.

After liposome processing, the non-viscous liposome suspension is converted to the desired gel form by titrating the pH of the suspension to a isoelectric point of the zwitterionic species. As mentioned above, the titration must be carried without significantly increasing the concentration of dissociable salts in the medium. This can be done by titrating with acids or bases which produce volatile salt components, such as certain ammonium salts, or which produce insoluble salts. Preferably, the titration is done by forming an initial liposome suspension in a medium containing low zwitterionic concentration, then titrating with a concentrated solution of the same zwitterionic compound, until the desired pH is reached.

It will be appreciated that a variety of liposome preparation methods, including reverse-phase evaporation and solvent-injection methods (Szoka, 1978, 1980), can be adapted for preparation of a liposome gel composition, using a low ionic-strength aqueous medium in the liposome formation step, in accordance with the invention.

The EGF/liposome gel composition of the invention can be prepared conveniently by a modified thin-film hydration method, as described above. Briefly, a lipid thin film is covered with hydration medium and allowed to hydrate, typically over a 15-60 minute period with agitation, with a low-conductivity buffer, as above. The final concentration of EGF/liposomes is at least 50 mg/g and preferably between about 50-200 mg/g composition. This method if illustrated in Example 1.

Alternatively, the liposome gel composition can be formed in two stages, involving initial liposome formation of a fluidic liposome suspension, by addition of the

aqueous buffer in an electrolytic condition (e.g., where the zwitterionic compound is not at its isoelectric point, and subsequent pH adjustment of the aqueous medium to a non-electrolytic state (isoelectric point of the medium), to produce the desired gel formation. The intermediate non-viscous liposome composition can be processed, as above, e.g., to achieve a desired liposome size range. This method is illustrated in Example 3.

The aqueous medium used in forming the composition may contain dissolved EGF, at a suitable concentration. The suspension formed in this manner includes encapsulated, liposome-adsorbed, and free EGF. Free EGF can be removed, if desired, by conventional methods, such as molecular sieve filtration or the like.

Alternatively, free EGF may be added to preformed liposomes at a suitable concentration, producing a suspension with liposome-adsorbed and free EGF. According to one aspect of the invention, it has been found that the in vivo release kinetics of EGF from EGF/liposomes containing absorbed EGF only is comparable for EGF/liposomes prepared to include both adsorbed and encapsulated EGF (Example 5).

From the foregoing, several advantages of the method of preparation of the liposome gel composition of the invention can be appreciated. The gel composition can be prepared at a low lipid concentration and thus is relatively inexpensive to manufacture. The final viscosity of the composition can be controlled by small changes in final ionic strength, produced either by addition or removal of ionic components, or by relatively small pH changes in a medium containing a zwitterionic buffer.

The liposome gel can easily be prepared and processed in a dilute form, for example to remove free drug, such as EGF, and to size and sterilize the liposomes, then brought to a final viscous state by pH adjustment.

Finally, as discussed in below, for preparation of an EGF\liposome gel composition, the EGF is adsorbed readily to the negatively charged liposomes in the EGF/-liposome composition, allowing the composition to be prepared simply by mixing free EGF with preformed liposomes.

E. Viscosity of the Gel Composition

The liposome gel composition of the invention is characterized by a high-viscosity gel-like consistency which is maintained at a low ionic strength, but which collapses as ionic strength is increased. This feature is illustrated in the study described in Example 2. Here liposomes containing equal-weight amounts of EPG, EPC, and cholesterol were prepared in a 2.3% w/v glycine buffer at isotonic pH (pH 6.0) buffer, as detailed in Example 1.

The mean viscosity for the samples was 13.3 x 10^3 Cps (centipoise) at 1.0 per second shear rate, characterized by a thick, relatively non-flowing gel consistency. With addition of NaCl to a concentration of only 0.05% w/v (about 8.5 mM), the material lost its gel-like properties, being quite fluid, with a mean viscosity of only about 2.7 x 10^3 Cps at 1 per second. Further relatively small decreases in viscosity were seen with further addition of NaCl to a final concentration of 0.2% w/v. The loss of viscosity at low NaCl concentration is seen in Figure 1.

The nature of the high viscosity gel composition can be appreciated from the liposome suspension models illustrated in Figures 2A-2C. The model shown in Figure 2A represents a liposome paste or concentrate containing a maximum concentration of lipid vesicles in an aqueous suspension medium. Empirically, viscous, paste-like lipid suspensions having a lipid concentrations of up to about 500 µg/ml can be produced, at which about 70% of the total aqueous volume is encapsulated.

The model of a liposome concentrate shown in Figure 2A assumes that at high lipid concentrations, and in the absence of surface charge effects, liposomes are able to form close packed suspensions in which the liposomes are densely packed, as indicated, being separated from one another only by a thin shell of ordered water (shown in dotted line). This model is consistent with the high percent of encapsulated water (up to 70%) observed in high-concentration liposome paste formulations.

Figure 2B shows a conventional liposome suspension containing about one-fifth the lipid concentration, e.g., 100 µg/ml. Assuming the suspension is composed of the same types of liposomes, more than 80% of the aqueous medium in the suspension would be non-encapsulated water, and each liposome would now be free to move through relatively large aqueous volume elements, as indicated. Because of this liposome mobility the suspension has a very low viscosity, i.e., is freely flowable.

Figure 2C shows the same low concentration of liposomes as in Figure 2B, but in a suspension formed in accordance with the invention in which the liposomes contain at least about 7 weight percent charged lipid component and a low-conductivity medium. The low lipid concentration of the suspension indicates that more than

80% of the total volume of the suspension is extra-liposomal water, i.e., non-encapsulated water. However, the high viscosity of the medium indicates that the liposomes are arrayed in packed spheres, as illustrated in Figure 2A.

These two assumptions are consistent with a model in which each liposome is surrounded by a relatively large spherical shell which contains a volume of up to several times that of the liposome, but which itself cannot be readily penetrated by the shells of neighboring liposomes. The thickness of the spherical water shells can be approximated from the following simplifying assumptions: (a) the maximum volume of liposome-encapsulated medium at a lipid concentration of 500 µg/ml is 70 percent; (b) in both high-and low-concentration suspensions, the uniform liposome sizes of about 2,000 Å; and (c) the total number of liposomes which is proportional to lipid concentration. The shell thicknesses given in Table 1 below can be calculated:

Table 1

Lipid Concentration (µg/ml)	Shell Thickness (Å)
100	1400
200	700
300	400
400	150
500	0

Since the immobilization of the liposomes in the dilute suspensions is assumed to be due to charge repulsion among charged, unshielded particles, the thicknesses of these

shells provide a rough estimate of the distance over which the charged liposomes exert an appreciable charge repulsion effect.

The concept of an electrostatic liposome shell is analogous to Debye length, which corresponds roughly to the distance over which the electrostatic field of an ion exerts an appreciable effect. Figure 3 shows a theoretical plot of Debye length as a function of concentration of a univalent electrolyte in solution. The rapid decrease in Debye length between 0-20 mM electrolyte closely mirrors the change in viscosity seen in Figure 1 over the same electrolyte concentration range, and strongly suggests the viscosity effect seen in the present invention is due to electrostatic barrier effects.

F. Properties of the EGF/Gel Composition

Viscosity

The EGF/liposome gel composition of the invention is characterized by a high-viscosity gel-like consistency which is maintained at a low ionic strength, but which collapses as ionic strength is increased. This feature is illustrated in the study described in Example 2. Here liposomes containing equal-weight amounts of EPG, EPC, and cholesterol were prepared in a 2.3% w/v glycine buffer at isotonic pH (pH 6.0) buffer, as detailed in Example 1, except that EGF was not added. The mean viscosity for the samples was 13.3 x 10^3 Cps (centipoise) at 1.0 per second shear rate, characterized by a thick, relatively non-flowing gel consistency. With addition of NaCl to a concentration of only 0.05% w/v (at about 8.5 mM), the material lost its gel-like properties, being quite fluid, with a mean viscosity of only about 2.7 x 10^3 Cps at 1 per second. Further relatively small decreases in viscosity were seen with further addition of

NaCl to a final concentration of 0.2% w/v.

EGF Binding to Negatively Charged Liposomes

According to one aspect of the invention, it has been found that EGF may be entrapped in negatively charged liposomes by surface adsorption, and that the binding affinity of EGF for the liposomes is effective to produce slow release of adsorbed peptide both in vitro and in vivo. In the binding study reported in Example 4, liposome gel compositions formed from either PC/PG (equal weight ratios) or PC/PG/cholesterol (equal weight ratios) were prepared as in Example 1. Increasing amounts of EGF (iodine radiolabeled) were added to aliquots of each of the two compositions, and the mixtures were allowed to equilibrate for one week at 4°C. The ratio of bound to free EGF was determined from total radiolabel measured before and after centrifugation, and these values were plotted as a function of amount bound, yielding the plots in Figures 4 and 5 for the EPC/EPG and EPC/EPG/cholesterol compositions, respectively. Affinity constants K_d were determined from these plots as described in Example 4. As seen from the two figures, the K_d values are in the range 1-2 x 10^{-5} molar for both compositions.

The number of EGF binding sites on the liposomes was determined from the x-axis intercept in the Figure 4 and 5 plots, along with the calculated K_d values, also as detailed in Example 4. From this, it was determined that at a peptide concentration of about 200 µg/ml, about 30% of the EGF is adsorbed at the lipid/water interface.

The adsorption of EGF to EPC/EPG and EPC/EPG/cholesterol monolayers was also examined in a lipid monolayer

system, also as detailed in Example 4. Briefly, the method measures the ability of EGF to interpenetrate the lipid monolayer, as evidenced by changes in the interfacial surface pressure as EGF is added to the monolayer.

Figure 7 is a plot of the interfacial surface pressure, π, as a function of lipid concentration for a EPC/EPG/cholesterol (equal wight ratios) lipid monolayers, as a function of lipid concentration. Similar plots were was made for EPC/EPG monolayers, and EPC/EPG/-cholesterol and EPC/EPG monolayers containing 40 µg/ml EPG, at each of several lipid concentrations. These plots were used to construct the graph of change in surface pressure due to the presence of 40 µg/ml EGF in the monolayer, as a function of surface pressure, for each of the two lipid compositions. This graph is shown in Figure 8.

Using linear regression analysis to extrapolate to the y-axis intercept it can be seen that the change in interface surface pressure produced by EGF in the EPC/-EPG/cholesterol composition is about 15 dynes/cm, and in the EPC/EPG composition, about 13.5 dynes/cm. The interface surface pressure attributable to EGF alone (no lipid interaction) is plotted in Figure 6, and is µ.6 dynes/cm at 40 µg/ml. Thus for both lipid compositions, the measured change in surface pressure due to EGF in the presence of lipid is greater than that produced by EGF alone, indicating that the peptide is interacting with the monolayer.

The greater EGF-induced change in pressure seen in the EPC/EPG/cholesterol composition indicates a greater degree of EGF interaction with the addition of cholesterol to EPC/EPG.

In vitro EGF Release Characteristics

The kinetics of release of EGF from EGF/liposome compositions prepared according to the invention were examined in a standard two-chamber percutaneous absorption cell, as detailed in Example 5. The samples placed in the donor cell were suspended in 25% human serum in isotonic saline, for passage across a membrane filter into a donor collector compartment which was continually perfused with 25% human plasma in saline.

Figure 9 shows release kinetics of EGF in the system for three independent kinetic studies. The mean halflife of EGF release, calculated from the slope of the availability of free EGF in the donor compartment, as a function of time, is about 1.8 hours.

The EGF available in the donor compartment from various EGF/liposome compositions were similarly measured. Figure 10-12 shows plots of EGF available in the donor compartment, as a function of time, from (Composition I) EPC/EPG liposomes with encapsulated EGF (Figure 10), (Compositions II) EPC/EPG/cholesterol liposomes with encapsulated EGF (Figure 11), and (Composition III) EPC/-EPG/cholesterol with adsorbed EGF (Figure 12). All three compositions contain free EGF, and thus also are expected to contain liposome-adsorbed EGF.

The model used to determine the half lives of EGF release from the liposomal formulations is discussed in Example 5. Briefly, the free EGF available in the donor compartment is determined from the measured rate of appearance of EGF in the receiver compartment, the rate constant K_b of the membrane, and the volume V_b of the external phase in the donor compartment. The calculated free available EGF in the donor compartment is then

plotted as a function of time, as seen in Figures 7-9. The half lives of EGF release during the slow phases is determined from the resulting plots.

The half lives determined from above are 14.1 hours for the EPC/EPG composition (encapsulated EGF); 10.1 hours for the EPC/EPG/cholesterol composition (encapsulated EGF); and 6.2 hours for the EPC/EPG/cholesterol composition (adsorbed EGF). It is clear that all of the liposome formulation enhanced the half life of EGF release in vitro severalfold over free EGF.

In vivo EGF Release Characteristics

The high-viscosity EGF/liposome compositions of the invention are effective to (a) remain physically localized at a site of injection or administration and (b) provide a source of therapeutic levels of EGF over a several-day period, as will now be demonstrated.

The enhanced retention of EGF in an EGF/liposome composition has been demonstrated with conjunctival placement of the various EGF compositions by sub-conjunctival-injection, and monitoring of levels of EGF retained at the conjunctival site over a several day period. The retention of radiolabeled EGF at a conjunctival site of administration, as a function of time after injection is shown in Figures 13-16 for free EGF (Figure 13), and EGF/liposomes composed of: (Composition I) EPC/EPG and containing free and encapsulated EGF (Figure 14), (Composition II) EPC/EGF/cholesterol and containing free and encapsulated EGF (Figure 15), and (Composition III) EPC/EPG/cholesterol and containing free and adsorbed EGF only (Figure 16). As seen, all of the EGF/liposome compositions give biphasic EGF release characteristics, indicating a burst of EGF released into the site of

administration, followed by a slow phase EGF release over a several-day period.

Table 3 in Example 6 gives the half-lives of EGF release, and the percent EGF released in the burst for free EGF and the three EGF/liposome compositions, calculated from the mean values of the data plotted in Figures 10-13. The half life of EGF retention was extended from 1 hour for free EGF to 14-35 hours for the liposomal compositions. Interestingly, and in contrast to the in vitro release kinetics observed, the largest half lives (32 and 35.6 hours) were obtained with Composition II and III (cholesterol-containing EPC/EPG liposomes), whereas the shortest half life (14 hours) was obtained with the Composition I. This discrepancy with the in vitro kinetics data may be due to the greater stability of cholesterol-containing liposomes in vivo, perhaps related to the reduced extent of lipid exchange which would be expected between liposomes and cells at the site of administration in the presence of cholesterol.

The long-term availability of EGF in the region of the EGF/liposomes is seen from the data in Table 4 of Example 6. For free EGF, substantially no EGF was available at the conjunctival site one day after administration. With the EGF/liposome formulations, more than 1% of the total EGF was available at the site 4 days after administration for Composition I, six days after administration, for Composition III, and seven days after administration, for Composition II.

From the foregoing, it can be appreciated that the degree of retention of EGF/liposomes at a site of administration can be selectively varied according to the amount of cholesterol included in the liposomes, at least over the range from about 0-33 weight percent.

The above results also indicate that liposome-adsorbed EGF in the EGF/liposomes is released from the liposomes in vivo at substantially the same rate as encapsulated EGF. This result confirms that EGF is tightly bound to negatively charged liposomes (containing at least 20 mole percent negatively charged phospholipid), and that an effective EGF/liposome formulation can be made by surface adsorption to liposomes.

G. Positively Charged Liposome Gel Composition

In another aspect, the invention includes a high-viscosity liposome gel composition for use either in applying lipid to a mucosal tissue, or in administering a liposome-entrapped drug to a mucosal surface tissue.

The composition includes a low-conductivity aqueous suspension medium having a selected pH between about 5.5 and 8.5, and between about 7-25 weight vesicle-forming lipids. The lipids contain between about 5-50 weight percent positively-charged vesicle-forming lipids, and (ii) the balance of neutral vesicle-forming lipids. Preferably the positively charged lipids are the type described above which include a spacer at least 3 atoms in length greater between the lipophilic portion or moiety of the lipid and the positively charged polar head group. These lipids provide enhanced liposome retention on mucosal surfaces. In particular, the positively charged lipid components in the composition preferably includes PE derivatives which are conjugated with basic amino acids, and/or amphophilic benzylyamine compounds, as described above. Lipid compositions 9-12 above are exemplary.

The compositions are formed substantially as described in Section D, where the aqueous hydration medium

may either be a low-conductivity medium, or a medium containing a zwitterionic compound which allows titration to a low-conductivity medium. Example 7 illustrates a PC/BDSA gel composition formed by solvent injection.

In one embodiment, the gel composition is formulated as a lipid supplement for treatment of dry eye. Preferred lipid compositions are detailed in U.S. Patent No. 4,818,537 for "Method of Treating Dry". The present differs from the earlier formulation in that high viscosity is produced by electrostatic effects rather than by high-viscosity polymers.

Alternatively, the positively charged liposome gel can be formulated to contain entrapped drug agents, for slow drug release from the liposomes.

II. Utility

A. Topical Administration

The liposome gel composition of the invention is useful as a moisturizing agent for application to dry or aging skin, and/or for applying cosmetic agents such as vitamin A, UV-blocking agents, or retin, to the skin. The gel is easily delivered from a tube or the like, is relatively non-greasy to the touch, and is clear when applied to the skin. One unique property of the gel is its ability to dissolve or melt over time as the gel becomes infused with salts on the skin. Thus, the viscous gel may be applied to the skin in gel form, but become quite fluidic as it is rubbed into the skin.

The gel may also be used as a drug delivery composition, for delivering a liposome-entrapped drug transdermally. The drug to be administered is typically a lipophilic drug, such as an anti-inflammatory steroid drug, which is entrapped in the gel liposome lipids at a con-

centration between about 1-20 weight percent. It will be appreciated that a charged lipophilic drug may be administered, where the charge on the compound contributes to the liposome surface charge.

The EGF/liposome composition may be used in application to burns and other skin wounds, to promote healing. Epidermal Growth Factor (EGF), is a widely distributed endogenous polypeptide (King). It is a powerful mitogen with high affinity receptors in both fibroblasts and epidermal keratinocytes, and has been shown to accelerate wound healing in vivo (O'Keefe; Knauer). The first 5-10 days after injury are the most critical period during which maximal differences are seen between EGF treated and untreated wounds. EGF application after this period produces no significant improvement over controls, since by this time re-epithelialization has already occurred in both groups.

For superficial wounds, local concentration of EGF can easily be maintained by applying the gel material directly to the skin or in a skin dressing. The material is preferably supplied in gel form from a tube or the like which can be easily applied to the skin or to a skin dressing. One unique property of the gel material, when applied directly to the skin as a film, is that salts in the skin will break down the gel structure, producing a fluid lipid dispersion as the material is rubbed in the skin.

B. Surgical Wound Administration

Because of its high viscosity, the gel composition is useful as a drug delivery vehicle for surgical wounds, where slow drug release over a several-hour to several-day period is required.

Due to its relatively short half-life of about one hour, (Buckley, 1987), loss of occupied receptors through turnover and a lag time of 8-12 hours to commit cells to DNA synthesis (Knauer), it has been necessary to apply EGF frequently to a wound to maintain effective local concentration during the critical period of initial wound healing (Buckley, 1987; Buckley, 1985; and Franklin, 1986). Thus, effective EGF therapy has required frequent or sustained application of the drug during the first several days of wound healing.

For surgical incisions and full thickness skin wounds requiring suture repair, frequent application of EGF is not possible and a sustained-release formulation of EGF must be used for these uses. Implanted sponges have demonstrated the advantages of sustained EGF release in an animal wound model (Buckley, 1985) but would not be suitable as a dosage form.

The EGF/gel composition of the invention substantially overcomes these limitations. In practice, the gel composition is applied incision area before suturing. The high viscosity of the of the material reduces loss of material from the incision site, and the slow release of EGF from the liposomes provides a therapeutic level of EGF at the site over a several-day healing period.

C. Ophthalmic Uses

The gel composition of the invention also provides a number of advantages for lipid or drug administration to mucosal tissue. For treatment of dry eye, where the gel serves as a source of lipid and moisture, the gel has the advantages of optical clarity, and enhanced retention due to high viscosity. Further, where the liposomes contain

positively charge lipid elements with charge spacers, as described above, the liposomes themselves have enhanced retention to corneal surfaces. The liposomes may also be used for drug delivery to the eye or other mucosal surface, with advantages of greater retention, i.e., less flow from, the site of application.

Figures 17A-17C illustrate surgical incision and incision repair step in a corneal replacement or transplant operation. An initial arcuate incision in the conjunctiva, illustrated in Figure 17A, allows the conjunctiva to be pulled away, exposing the underlying episclera and cornea. A second arcuate cut in the cornea, shown in Figure 17B, allows the cornea to be pulled back to provide access to the lens (not shown). After surgical removal or replacement of the lens, the cornea is first closed by stitching, seen at 20 in Figure 17C, followed by closure of the conjunctiva by stitching, indicated at 20. Post-operative healing involves healing of the two incisions, and regrowth of the episclera layer between the conjunctiva and cornea.

Figures 18A-18C illustrate the use of the EGF/liposome composition of the invention to promote healing of the above-described ophthalmic surgery. Figure 18A shows a cross section through an outer portion of the stitched cornea and conjunctiva, as seen in perspective in Figure 17C. After stitching, the space between the conjunctiva and cornea is filled with an EGF/liposome composition, by inserting a needle through a region of the stitched incision in and injecting the composition into the episclera space. As illustrated in Figure 18B, the material may be injected until a slight bulging of the conjunctiva is produced.

The injected material remains in place, over a several-day period, by virtue of its gel or paste-like consistency. EGF is released into the surrounding area, promoting healing of both stitched incisions and regrowth of the episclera over an extended healing period.

Preliminary studies conducted in support of the present invention have examined the effect of viscous EGF/liposomes on ophthalmic incision repair in an animal model system. Briefly, it has been found that a viscous EGF/liposome composition provides greater wound repair, as measured by the strength of the repaired incision several days after treatment, than empty liposomes.

According to another important advantage, the gel composition of the invention combines high viscosity with low lipid concentration, so that the material is relatively inexpensive in terms of materials cost. Further, additives, such as high molecular weight polymers, colloids and the like, are avoided.

The following examples are intended to illustrate various compositions, methods of preparations, and characteristics of the present invention. The examples are in no way intended to limit the scope of the invention.

Example 1

Preparation of EGF/Liposome Compositions

EPG was purchased from Avanti Polar Lipids (Birmingham, AL) and EPC was purchased from Asahi Chemical Company (Tokyo, Japan). Cholesterol was from Croda, Inc. (New York, NY) and α-tocopherol (Vitamin E) from Hoffman - La Roche (Nutley, NJ). Aminoacetic acid (glycine) was from J.T. Baker (Philipsburg, NJ).

EGF from yeast (Chiron) was a gift of Ethicon, Inc. (Somerville, NJ). ^{125}I-labeled rh-EGF (3-[^{125}I] iodotyrosyl

human recombinant epidermal growth factor was purchased from Amershal Corporation (Arlington Heights, IL). ^{125}I rh-EGF was shipped the day of iodination and used only for the first four weeks following iodination.

Liposomes were prepared by thin film hydration of a dehydrated lipid mixture containing one of the following lipid mixtures. Composition I: EPG/EPC/α-tocopherol (1/1/0.03, w/w/w) and Composition II: EPG/EPC/cholesterol/ -tocopherol (1/1/1/0.03, w/w/w/w). The lipids were dissolved in chloroform:methanol (2:1) and a total of 33 g of lipid were added to a round bottom flask and dried in vacuo to a thin film. To this film was added 267 ml of hydration buffer containing 2.3% (w/v) glycine, pH 6.0. Hydration was carried out for 1-2 hours with swirling. The material had a stiff, gel-like consistency.

The materials were prepared in a biological cabinet using sterilized equipment, filter-sterilized lipid, and filter-sterilized aqueous solutions to keep the bioburden as low as possible. The vesicles were prepared by thin-film hydration in a 2.3% glycine buffer. The resulting liposome dispersion was injected by extrusion through a Gelman Acrodisc into 1 or 10 ml plastipak syringes which were wrapped in aluminum foil and labeled. An aliquot of the liposome gel was set aside, and "collapsed" back into a lotion by the addition of concentrated saline.

Samples were assayed for rh-EGF concentration, total lipid phosphate, cholesterol content, pH, viscosity, osmolarity, particle size and pyrogen levels (Table 2). Mean diameters were assayed using the Nicomp laser particle sizer.

TABLE 2

Characterization of EGF Compositions

Assay (units)	rh-EGF-Loaded "Lotion"	rh-EGF-Loaded "Gel"	Placebo "Lotion"	Placebo "Gel
rh-EGF (µg/gm)	192	197	0	0
Total Lipid Phosphate (µmol/gm)	91.1	100.4	87.0	87.0
Cholesterol (mg/gm)	31.6	31.6	34.3	32.2
Buffer pH	6.1	6.1	6.0	6.0
Osmolarity (mOsm)	326	313	319	305
Viscosity (Cps) [c]	2,750	17,700	3,600	20,500
Nicomp Mean Diameter (nm)	630	713	644	666
LAL Pyrogen Test	Pass	Pass	Pass	Pass

Example 2

Viscosity of the EPG Gel Liposome Composition

Five separate batches composed of EPG/EPC/ cholesterol/alpha-tocopherol (1/1/1/0.03, w/w/w/w) liposomes were prepared as described in Example 1. The viscosity of each of the batches was determined (a) without addition of NaCl, and after addition of (b) 0.05%, (c) 0.1, and (d) 0.2% by weight NaCl. At each salt concentration, the mean viscosity of the compositions tested was determined. The measured values, expressed as extrapolated Cps at 1

per second shear rate, are shown in Figure 4 and in Table 3 below.

Viscosity was determined using a Brookfield DV-II cone/plate viscometer. Viscosity readings were made at all relevant spindle speeds. Spindle speeds were converted to the shear rate. Plots of log (viscosity) versus log (shear rate) were prepared from which the viscosity at a shear rate of one reciprocal second was extrapolated.

Table 3

% NaCl	Mean Viscosity (Extrapolated Cps at 1 sec. shear rate)
0.0 %	13.3×10^3
0.05%	2.7×10^3
0.1 %	1.5×10^3
0.2 %	0.8×10^3

The mean viscosity of the composition in the absence of NaCl corresponds to a stiff, gel-like consistency. As seen, addition of only a slight amount of salt reduces the viscosity severalfold, producing a thinner, lotion-like consistency.

Example 3

Processing EPG Liposome Gel Composition

Liposomes were prepared by thin film hydration of a dehydrated lipid mixture containing EPG/EPC/cholesterol/ -tocopherol (1/1/1/0.03, w/w/w/w), as described in Example I, except that the hydration buffer used to

produce the liposomes contained 50 mM glycine, adjusted to pH 8. The liposome suspension was highly fluidic.

The suspension was sized by extrusion by multiple through a 0.2 micron polycarbonate membrane. The sized liposomes were then sterilized by filtration through a 0.25 micron depth filter. To this sterilized material was added 1/10 volume of sterilized 10X glycine, adjusted to produce a final pH of 6.0. The final suspension had a stiff, gel-like consistency.

Example 4

Adsorption of EGF to Liposomes

A. Scatchard Analysis

The affinity of EGF binding to liposomes and the number of binding sites in the Example 1 liposomes can be determined from Scatchard analysis of the binding of radiolabled rh-EGF to the liposomes.

Liposomes formulations I and II in Example 1 were prepared by hydration of the thin lipid film with 2.3% glycine (w/w), as described in Example 1. The formulations were sized (three passes for each pore size) sequentially through 5.0 μm and then 1.2 μm polycarbonate filters, then extruded through a 0.4 μm Nuclepore filter.

Five μCi of ^{125}I-rh-EGF were added to 2 ml of rh-EGF, 1 mg/ml concentration in glycine buffer. Aliquots of this iodinated stock solution were added to duplicate, 1 ml samples of the liposome preparation also containing five final rh-EGF concentrations ranging from 6 to 100 μg rh-EGF per ml liposomes. The resulting preparations were allowed to come to equilibrium by incubation for one week at 4° C.

After equilibration, known volumes of the preparations were removed for gamma counting. A known volume of the bulk of the remainder of each sample was centrifuged for two hours at 40K RPM and 4° C. Known volumes of the clear supernatant ("free" EGF) were removed for gamma counting. Any remaining supernatant was removed and discarded. Each liposome pellet was resuspended in 1.2 ml of 1% (w/w) Triton X-100 and vortexed. All samples were then assayed for gamma counts, and the actual CPM per ml were determined.

The ratio of bound/free was determined for each sample as:

Ratio = [bound]/([bound plus free]-[bound]) (Eqn. 2)

Values of [bound]/[free] versus [bound] were plotted and the data fit to a linear least squares regression. Figures 4 and 5 show the plots for the I and II formulations, respectively. K_d, the binding constant of the peptide on the liposomes was determined from the slope of the regression line, which was taken to be $1/K_d$. Confidence intervals of K_d were calculated according to known methods (Tallarida).

The results seen in Figures 4 and 5 indicate that there is no significant difference between the two formulations as to affinity constants, which is calculated as about 1-2 x 10^{-5} M for both formulations. The K_d for liposomes is several orders of magnitude less than that for cultured fibroblasts (2 - 4 X 10 -10 M) (Buckley, 1987).

The number of binding sites was determined from the X-axis intercept of the of the regression line, which was taken to be equal to [binding sites]K_d (Scratchard). The EPC/EPG formulation had 0.8 µg EFG binding sites per mg

lipid, and the EPC/EPG/cholesterol formulation had 1.4 µg EGF binding sites per mg lipid. The number of binding sites is actually the number of binding sites on the external face of the lipid bilayer. Thus, for large unilamellar vesicles, the actual number of binding sites would be twofold greater than for multilamellar or oligolamellar preparations like these, at least threefold greater. The lipid concentration used is sufficient to potentially adsorb all the rh-EGF.

Knowing the estimates for K_d and the number of binding sites, it can be calculated that at this lipid concentration and at a peptide concentration of about 200 ug rh-EGF/gm formulation, about 30% of total rh-EGF is adsorbed at the lipid/water interface.

B. Surface Pressure Measurements

Adsorption of native rh-EGF lipid monolayers to lipid/water interfaces can be evaluated by measuring surface tension of lipid monolayers spread on an rh-EGF-containing aqueous subphase. The methodology of Weiner and coworkers (Schwinke) was used to rank order different lipid monolayer compositions with respect to the enhanced ability of a peptide to interpenetrate a given monolayer. Distilled water adjusted to pH 6.0 was used as the subphase in these pilot experiments. Other experiments done in 2.3% glycine as buffer gave the same results.

Surface tension measurements were made on a CSC Scientific Model 70545 DuNouy tensiometer (Fairfax, VA). Briefly, a new lipid monolayer was spread from a hexane/-ethanol (95/5, v/v) solution for each determination. Surface pressure (π) was determined as the difference of the surface tension of test monolayer of subphase minus

the surface tension of subphase alone. Delta π is the difference between mean monolayer surface pressure in the presence and absence of rh-EGF. All data points are the mean of at least duplicate determinations.

Figure 6 is a plot of surface pressure π of aqueous EGF measured as a function of EGF concentration. As illustrated in the figure, rh-EGF is a surface active peptide having a limiting pressure of 12.6 dynes/cm as determined from a double reciprocal transformation of this graph. Surface pressure changes also be monitored as a function of lipid concentration. As an example, EPG/EPC/Chol (1/1/1, w/w/w) monolayers displayed a limiting pressure of 50.9 dynes/cm in the absence of rh-EGF in the aqueous subphase (Figure 7). This limiting pressure (also called the collapse pressure) is considered to be the equilibrium pressure of liposome bilayers (MacDonald).

The ability of the peptide to interpenetrate the lipid layer can be determined, as indicated above, by measuring interfacial surface pressure in the presence and absence of peptide at several lipid concentrations. The difference in surface pressures (delta π) is plotted versus the surface pressure in the absence of peptide.

In Figure 8, data for EPG/EPC (1/1, w/w) and EPG/-EPC/Chol/a-toc (1/1/1, w/w/w/) are plotted. The rh-EGF concentration in the subphase was 40 ug/ml. A linear regression analysis was carried out on the data to extrapolate to the y-axis intercept (limiting π) (Wiener). The fact that this value, e.g., 15 dynes/cm for the EPC/EPG/cholesterol formulation, is greater than the peptide's own limiting pressure in the absence of lipid monolayer, is evidence that the peptide is penetrating

the lipid monolayer. That is, the observed effect is not merely competition for the surface by two different surface-active molecules (Schwinke). Furthermore, the formulations may be rank-ordered according to the magnitude of the extrapolated peptide/lipid limiting π.

Based on this analysis, both Composition I (solid circles in Figure 8) and Composition II (open triangles in Figure 8) show rh-EGF adsorption to the lipid. The greater extrapolated value of delta π for the EPC/EPG/-cholesterol formulation suggests that this composition formulation shows a greater degree of peptide/bilayer interaction than the EPG/EPC (1/1, w/w) formulation.

Example 5

In vitro EGF Release Kinetics from EGF/Liposomes

Liposome formulations were evaluated in vitro by release rates of 125 I-rh-EGF into the receiver compartments of percutaneous absorption cells that were continuously perfused with 25% pooled human plasma/saline.

A conventional two-chamber absorption cell, using a bath temperature of 37°C, was employed (Bronaugh). A 25 mm, 0.08 µm diameter Nuclepore (Pleasanton, CA) polycarbonate filter was used to separate the donor from the flow-through acceptor compartment. This pore size gave the shortest half life of free EGF passage --about 1.8 hours-- without detectable passage of intact liposomes through the membrane. The ^{125}I-rh-EGF preparation was mixed with an equal quantity of 25% pooled human plasma/isotonic saline and 200 µl aliquots were immediately applied to the donor compartments. Parafilm was used to occlude the donor compartment and prevent evaporative loss. The perfusate was collected by fraction collector

into scintillation vials and counted in a Packard TriCarg 20000. rh-EGF flux was calculated from the specific activity (DPM per µg peptide). Clearance rate half-lives were determined for individual percutaneous absorption cells as described below.

Figure 9 shows the available radiolabeled rh-EGF in the donor compartment of three Bronough cells, as a function of time, for an rh-EGF solution. In this single phase system, it is possible to determine the concentration of peptide remaining in the donor compartment by subtracting cumulative peptide in the measured in the receiver compartment from total peptide applied to the donor compartment. The half-life of EGF release determined from the mean of the three EGF curves, is about 1.8 hours. Since the membrane is rate-limiting for EGF transfer from the donor to receiver compartment, the curve can also be used to calculate K_b, the rate constant of the membrane.

The determination of drug-release halflife from the pool of free EGF available in the donor compartment is more complicated, due to the fact that in EGF/liposomes, only EGF which is located in the external aqueous phase is potentially bioavailable. A model proposed by Chowhan et al for solute flux from a liposomal carrier is represented by the following equation:

$$C_{II} = (dAs/dt) / (K_b * V_b) \qquad \text{(Eqn.1)}$$

where, C_{II} is the concentration of the solute of interest in the external aqueous phase, dAs/dt is the rate of solute appearance in the receiver compartment during the time period of interest, K_b is the rate constant of the membrane separating donor and receiver compartments, and V_b is the volume of the external phase in the donor compartment. The rate of solute appearance in the donor

compartment was determined, as above, by assaying the amount of radiolabel in the donor compartment over the efflux period. K_b was determined from the free drug efflux study above, and V_b can be estimated to within about 10%. Using the equation above, the C_{II}, the available free EGF in the donor compartment, was calculated as a function of time. Figure 10 shows plots for seven Bronaugh cells, where the heavier line represents the overlap of two or more plots.

The release profile of all liposome formulations was best modeled by bi-exponential fit. The slow-phase $t_{1/2}$ of Composition I EPG/EPC/a-toc (1/1/0.03, w/w/w) MLVs with free and entrapped rh-EGF was about 14.1 hours. "Free" rh-EGF in the external phase of the donor compartment was maintained above a concentration of 0.063 µg EGF/ml (the lower threshold of therapeutic activity) for over 50 hours.

Figure 12 shows similar plots from five Bronaugh cells of the availability of free EGF in the donor cell, as a function of time, for an EGF/liposome composition having the Composition II formulation EPG/EPC/Chol/a-toc (1/1/1/0.03, w/w/w/w). As with the composition above, the liposomes contained liposome-encapsulated EGF, as well as liposome-adsorbed and free EGF.

The available, i.e., free EGF, in the donor compartment was determined as above. From the plots, a mean halflife of about 10.1 hours for the slow phase of the release kinetics was observed. Thus, free rh-EGF displayed a somewhat shorter $t_{1/2}$ than for the liposome formulation lacking cholesterol, although the two halflives are not statistically different.

In another study, rh-EGF was added to pre-formed EPG/EPC/Chol/a-toc (1/1/1/0.03, w/w/w/w) MLVs to give an

initial external phase peptide concentration in the vicinity of 100 ug/gm formulation. The slow phase $t_{1/2}$ of this formulation (Composition III) was significantly longer (6.2 hrs, Figure 12) than that of the free rh-EGF solution tested in the same model, and somewhat less than the above liposome compositions containing both encapsulated and liposome-entrapped EGF. The data indicate that release of adsorbed EGF from liposomes is rate limiting and occurs with roughly the same release kinetics as EGF which is both encapsulated in and adsorbed to the liposomes.

Example 6

In vivo EGF Release Kinetics from EGF/Liposomes

The EGF/liposome compositions tested are given in Section 1B above. All liposome formulations consisted of unsized multilamellar vesicles containing egg phosphatidyl glycerol and partially hydrogenated egg phosphatidyl choline, prepared substantially as in Example 1. All formulations contained glycine buffer (2.3%) as a gelling agent and a-tocopherol (0.1%) as an antioxidant. All formulations contained ^{125}I-h=EGF (0.01 uCi/ug) as a radiolabel, and some contained a small amount of tritiated cholesterol (<0.1% of total lipid) as a formulation marker. Free EGF and Composition I contained Thimerosal (0.1%) and dimethylene-triaminetetraacetic acid (DTPA) (0.1%), but Compositions II and III did not. In addition Compositions II and III were prepared aseptically in an attempt to minimize irritation following administration.

Compositions II and III had the same lipid composition but were loaded differently. EGF was encapsulated into Composition II liposomes during formation, and therefore contained entrapped drug. EGF was added to the

liposomes of Composition III after formation, and was therefore considered adsorbed to the liposome surface. Total EGF was adjusted to give similar quantities of EGF in the external aqueous phase in both compositions II and III.

Table 4

CHARACTERISTICS OF EGF FORMULATIONS

	FORMULATION			
	Free EGF	Liposome I	Liposome II	Liposome III
Type	Free	Adsorbed and Entrapped	Adsorbed and Entrapped	Adsorbed
EPC, mg/g	0	130	35	35
EPG, mg/g	0	130	35	35
Cholesterol mg/g	0	0	35	35
EGF, ug/g	100	100	100	40
DTPA + Thimerosal	Yes	Yes	No	No
Aseptic Process	No	No	Yes	Yes
pH	7.0	6.05	6.02	6.02
EGF Dose, ug/kg body wt.	2.3	2.1	1.6	0.63

Formulations of free or liposome associated ^{125}I EGF were administered by subconjunctival injection to female New Zealand White Rabbits (2-3 kg). Conscious rabbits were placed in a bag restrainer, and 1-2 drops of Ophtaine 0.5% Ophthalmic Solution were administered to the eye as a local anesthetic 1 minute prior to the injec-

tion. A small fold of conjunctiva superior to the cornea was raised with forceps and 50 ul of the formulation injected slowly through a 27 ga needle using a calibrated glass microsyringe (Hamilton Co., Reno, NV). Both left and right eyes were used on separate days in some rabbits but no eye was used more than once during the study. Eyes were monitored for signs of leakage, inflammation or irritation. Small blebs formed following injection which tended to migrate slowly downward over a period of days. A liposome encapsulated dye appeared to remain localized in the eye for over 5 days following injection in one rabbit, thus indicating the ability of a formulation to remain physically localized at the injection site during the study.

The amount of radioactivity remaining in the injected eye was measured for up to 96 hours after injection (until at least 90% of the initial radioactivity had disappeared). An external NaI crystal detector/ratemeter (The Nucleus Model L) was placed directly over the eye and radioactivity measured for 2 minutes and recorded on a chart recorder. A standard curve was prepared by serial dilutions of the dose solution to calibrate and demonstrate the linearity of the detector response. The mean chart deflection during each recording was taken as the measurement at that time point. The first reading was taken immediately after injection ("zero time") and subsequent measurements were converted to a percentage of this initial amount.

Individual radioactivity versus time data were recorded for each animal, and the mean values for each animal group (N= 4-6) were calculated. These data are plotted in Figures 13-16 which show the remaining ^{125}I-rh-EGF in conjunctiva as a function of time following sub-

conjunctival injection of (a) free EGF (Figure 13), (b) Composition I EGF/liposomes (Figure 14), (c) Composition II EGF/liposomes (Figure 15), and (c) Composition III EGF/liposomes (Figure 16). As seen from Figure 11, unencapsulated EGF disappeared rapidly from rabbit eyes after subconjunctival injection (Fig.1). This disappearance was first-order and had a half life of 1.0 hours. Less than 15% of the dose remained in the eye after 3 hours, with only 1 x 10^{-5}% expected to remain after 24 hours. In contrast, EGF administered in liposome formulations disappeared much more slowly from the eye. Formulation I (EPC/EPG) exhibited a small initial burst disappearance followed by first-order disappearance with a half-life of 14.1 hrs (Figure 11). Formulations II and III (EPC/EPG/CH) both exhibited a larger initial burst followed by a slower first-order disappearance (Formulation II half-life: 32 hrs, Formulation III half-life: 35.6 hrs).

Each plot was used to calculate first order rate constants for EGF disappearance from the eye for each formulation by a non-linear least squares fitting method (RSTRIP, MicroMath, Salt Lake City, UT). Where a significant initial burst release was observed, only the log-linear portion of the curve after the rapid initial phase was fitted. The calculated rate constants, half-life, and % of dose in the burst release are given in Table 5 below.

Table 5

IN VIVO PERFORMANCE OF LIPOSOME EGF FORMULATIONS

	Free EGF	FORMULATION I (No cholesterol)	II (entrapped)	III (adsorbed)
Rate constant hr^{-1} [a]	0.69	0.049	0.022	0.019
Half-life, hrs	1.0	14.1	32.0	35.6
Burst Release % of Dose	N/A	15.0	43.0	57.0

(a) Estimated from non-linear least squares fit of log-linear portion of disappearance curve following initial burst (see text).

Since the half-line of disappearance of free EGF was 1.0 hr, it is reasonable to assume that the release of EGF from the liposomes is the rate limiting step in the prolonged disappearance of EGF from the eye observed in liposome-containing formulations, and that observed disappearance half-lives approximate half-lives of EGF release from the liposomes. From the first order rate equation:

$$A_t = A_0 * e^{-kt} \quad \text{or} \quad \ln(A_t) = \ln(A_0) - kt$$

where $k = 0.693 / t_{1/2}$, the amount of EGF lost from the eye during any day can be calculated. For example, the amount of EGF lost on day two (24-48 hrs) for Formulation I ($k = 0.049\ hr^{-1}$, $A_0 = 85\%$) is founded by taking the difference between A_{24} and A_{48} where $A_{24} = 85 * e^{-0.049*24} = 26.2$ and $A_{48} = 85 * e^{-0.049*48} = 8.09$. This difference is 18.1% of the total dose administered.

Using the first-order rate constants from Table 5, and estimating A_0 as the total dose minus burst, the

percentage of the administered EGF dose lost on each day was estimated (Table 6).

Table 6

	FORMULATION			
	FREE EGF	I (No cholesterol)	II (entrapped)	III (adsorbed)
%Lost: (a)				
Day 1	100	73.8	63.6	71.8
Day 2	0	18.1	14.3	10.5
Day 3	0	5.6	8.7	6.6
Day 4	0	1.7	5.3	4.1
Day 5	0	0.53	3.2	2.6
Day 6	0	0.16	1.9	1.6
Day 7	0	0.05	1.2	1.0

(a) Based on first order rate constants shown in Table 5 (see test for details of calculations). Day 1 values include initial burst. Values are % of total dose administered.

All three EGF/liposome compositions gave at least 1% per day for the first four days, and Composition II and III continued to deliver this amount of EGF for up to 7 days.

The addition of cholesterol reduced the rate of EGF disappearance in liposomes, but increased the initial burst release (up to 57% in the first 40 minutes). This burst effect may be due to release of adsorbed drug from the outer bilayer(s) during initial in vivo destabilization, but it is not clear why burst release was lower

with the cholesterol-free formulation (I). The effect of a burst release is to rapidly elevate local drug concentration after injection. Therefore, a certain degree of burst release may be advantageous in establishing rapid onset of action. Even though they lost more of their contents early, the cholesterol-containing formulations (II & III) were able to deliver more drug on days 3 to 7 due to their slower release rate.

Local erythema and edema were observed in some rabbits following administration. All of the rabbits receiving free EGF displayed redness at 90 minutes and increasing edema by the end of the study (3 hours), at which time they were treated with ophthalmic ointment. Following injection of Formulation I, all eyes appeared reddened at 90 minutes, erythema and edema increasing to a maximum at 6 hours and then subsiding. Free EGF and Composition I contained Thimerosal and DTPA, and were not prepared aseptically. Composition II and III were subsequently prepared aseptically without Thimerosal or DTPA. These formulations caused mild erythema in 6 of 8 rabbits, but edema was observed in only one animal, indicating reduced irritation compared with the first two formulations.

Both Compositions II and III appeared to release EGF at approximately the same rate despite the fact that Composition II contained entrapped and absorbed drug while Composition III contained only adsorbed drug. This may indicate that EGF (an amphipathic peptide) is strongly adsorbed to the bilayer, as discussed in Example 3 above, and that the rate limiting step in its release involves desorption from its membrane associated state, rather than "leakage" through the lipid barrier.

Example 7

Positively Charged Liposome Gel Composition

Fully hydrogenated soy PC (HSPC) were obtained from American Lecithin Company (Atlanta, GA). Benzyldimethylstearylammonium chloride (BDSA) was obtained from Aldrich Chemical Company (milwaukee, WI).

A lipid mixture containing 32 g HSPC and 4 g BDSA was dissolved in 38 ml ethanol, at about 60° C. This lipid solution was then injected slowly, with stirring, into 500 ml of distilled water, also at 60° C. The liposome suspension which was produced was cooled to room temperature, resulting in a substantially non-flowing gel.

Although the invention has been described with respect to particular embodiments, methods, and applications, it will be apparent that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A high-viscosity liposome gel composition comprising

(a) a low-conductivity aqueous suspension medium having a selected pH between about 5.5 and 8.5, and

(b) suspended in the medium, at a concentration of between about 7 to 25 weight percent total lipid, liposomes composed of (i) between about 5-50 weight percent charged vesicle-forming lipids which contribute a common net charge to the outer surfaces of the liposomes, at the selected pH, and (ii) the balance of neutral vesicle-forming lipids.

2. The gel composition of claim 1, wherein the aqueous medium contains a zwitterionic compound whose isoelectric point is substantially at the selected pH.

3. The gel composition of claim 2, wherein the zwitterionic compound is a neutral amino acid.

4. The composition of claim 3, wherein the concentration of zwitterionic compound is such as to produce a substantially isotonic medium.

5. The composition of claim 1, wherein the charged vesicle-forming lipids phosphatidylglycerol, and the neutral vesicle-forming lipids include phosphatidylcholine.

6. The composition of claim 5, which further includes liposome-entrapped epidermal growth factor, and

wherein the concentration of phosphatidylglycerol in the liposomes is between about 10-50 weight percent.

7. The composition of claim 5, wherein the liposomes are composed of between 20-40 weight percent each of phosphatidylglycerol, phosphatidylcholine, and cholesterol.

8. The composition of claim 1, for use in applying liposomes to a mucosal tissue, wherein the charged vesicle-forming lipids are positively-charged components.

9. The composition of claim 8, wherein the charged lipids include a phosphatidylethanolamine conjugate prepared by derivatizing phosphatidylethanolamine with a basic amino acid.

10. The composition of claim 8, wherein the charged lipids includes a benzylamine lipid.

11. An epidermal growth factor (EGF)/liposome composition having a gel-like consistency, comprising

(a) liposomes containing between 20-50 mole percent each of phosphatidylcholine, phosphatidylglycerol, and cholesterol, at a total lipid concentration of between about 50-200 mg/g composition;

(b) a low-conductivity aqueous medium containing a zwitterionic compound whose isoelectric point is between about pH 6-8; and

(c) liposome-entrapped EFG, at a concentration between about 0.5 - 5 μg/ml.

12. A method of preparing a liposome gel composition, comprising

mixing a lipid composition containing between about 5-50 weight percent charged vesicle-forming lipids having a common charge at a selected pH between about 5.5 and 8.5, and the balance of a neutral vesicle-forming lipids with a low-conductivity aqueous suspension medium, at a total lipid concentration of between about 7-25 weight percent.

13. The method of claim 12, wherein the aqueous medium contains a zwitterionic compound whose isoelectric point is between about pH 5.5 and 8.5.

14. The method of claim 13, wherein said mixing includes adding the lipid composition to an aqueous medium whose pH is substantially different from the isoelectric point of the zwitterionic compound, thereby to form a substantially fluidic liposome suspension, and adjusting the pH of the suspension to the isoelectric point of the zwitterionic compound, to produce the desired gel-like suspension.

15. The method of claim 14, which further includes processing the relatively fluidic liposome suspension to achieve a desired liposome size change prior to said adjusting.

16. A method of forming an epidermal growth factor (EGF)/liposome composition having a gel-like consistency, comprising

(a) providing a lipid mixture containing neutral phospholipid, between 10-50 weight percent negatively charged phospholipid and EGF; and

(b) suspending (i) the lipid mixture to a final lipid concentration between about 50-200 mg/ml, and (ii) EGF, to a final EGF concentration of between about 0.1 and 10 μg/ml, in a low-conductivity aqueous medium containing a zwitterionic compound having an isoelectric point between pH 5.5-8.5.

17. The method of claim 16, wherein said peptide is added after formation of liposomes in the aqueous medium, and the polypeptide is predominantly adsorbed to the liposome surfaces.

18. A method of treating a wound or surgical incision with a sustained release dose of epidermal growth factor (EGF),

providing a high-viscosity liposome composition comprising (a) EGF/liposomes (i) containing neutral phospholipid, at least about 10 weight percent negatively charged phospholipid, and liposome-entrapped EGF, and (ii) having a lipid concentration of less than about 200 mg/g composition and a pH between 5.5 and 8.5 and (b) means for imparting a high composition viscosity selected from the group consisting of (i) an low-conductivity aqueous medium containing a zwitterionic compound having an isoelectric point between 5.5 and 8.5; and (ii) empty liposomes which are substantially free of negatively charged phospholipid and liposome entrapped EGF, and

applying the composition to the wound or incision site.

19. The method of claim 18, for treatment of surgical incision, wherein the composition is applied between surgical incision layers.

20. The method of claim 19, for treatment of ophthalmic surgical incisions involving both corneal and conjunctiva incisions, wherein the composition is injected to the space between the cornea and conjunctiva, after resuturing the conjunctiva.

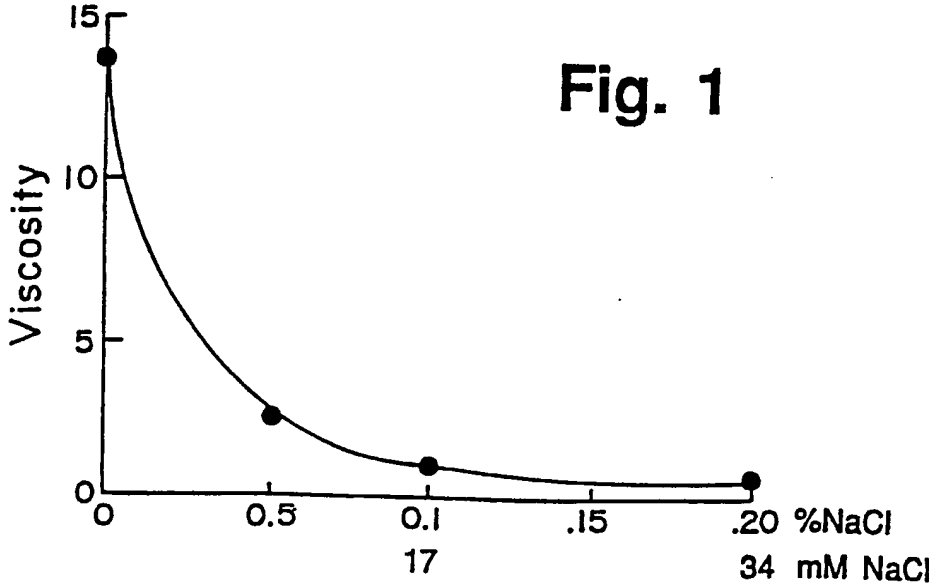


Fig. 1

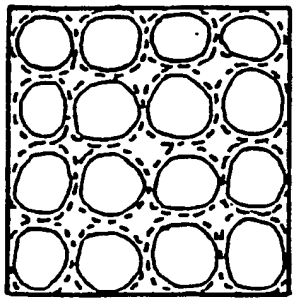


Fig. 2A

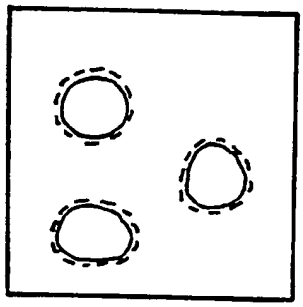


Fig. 2B

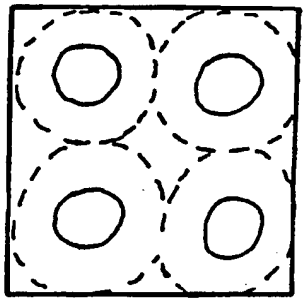


Fig. 2C

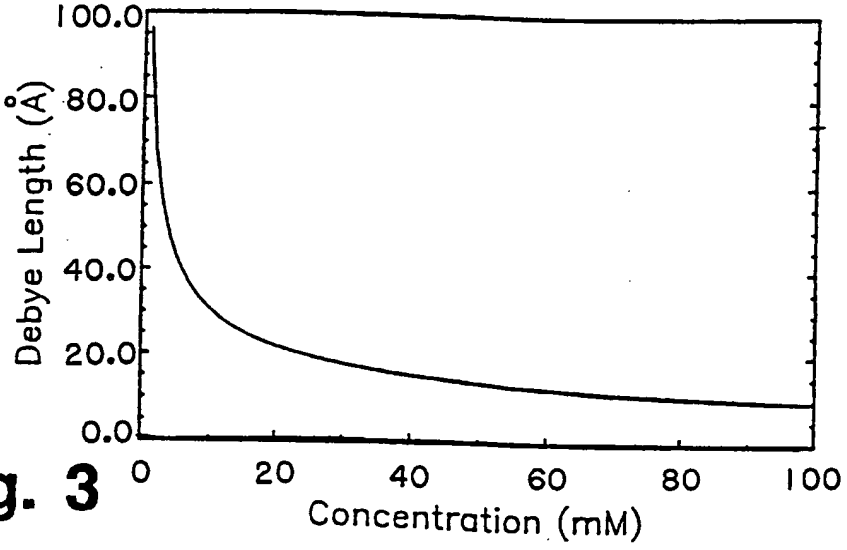
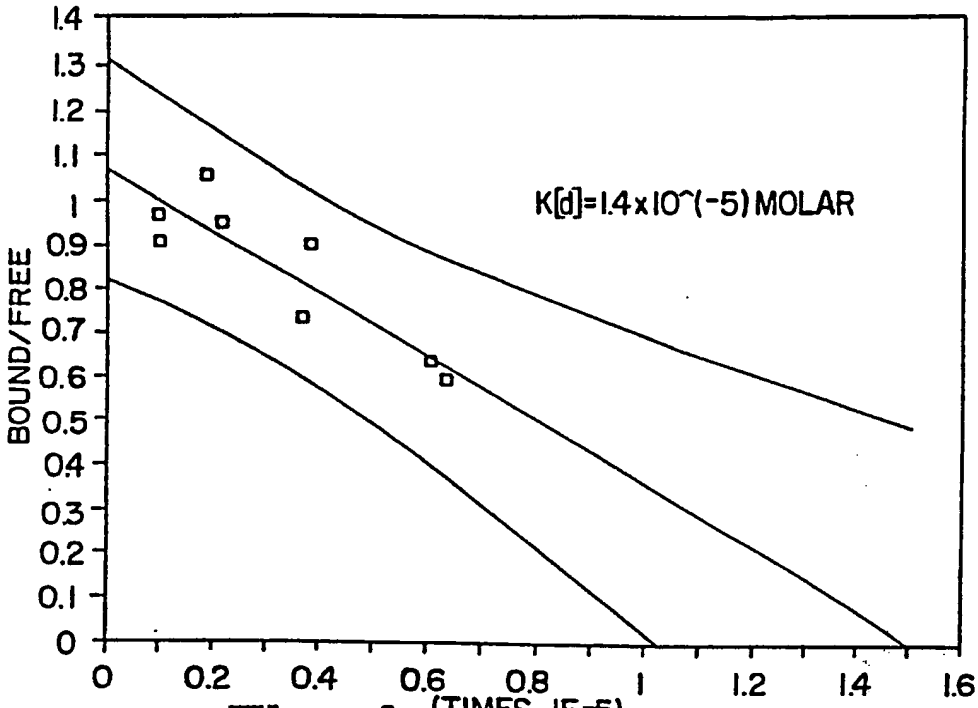


Fig. 3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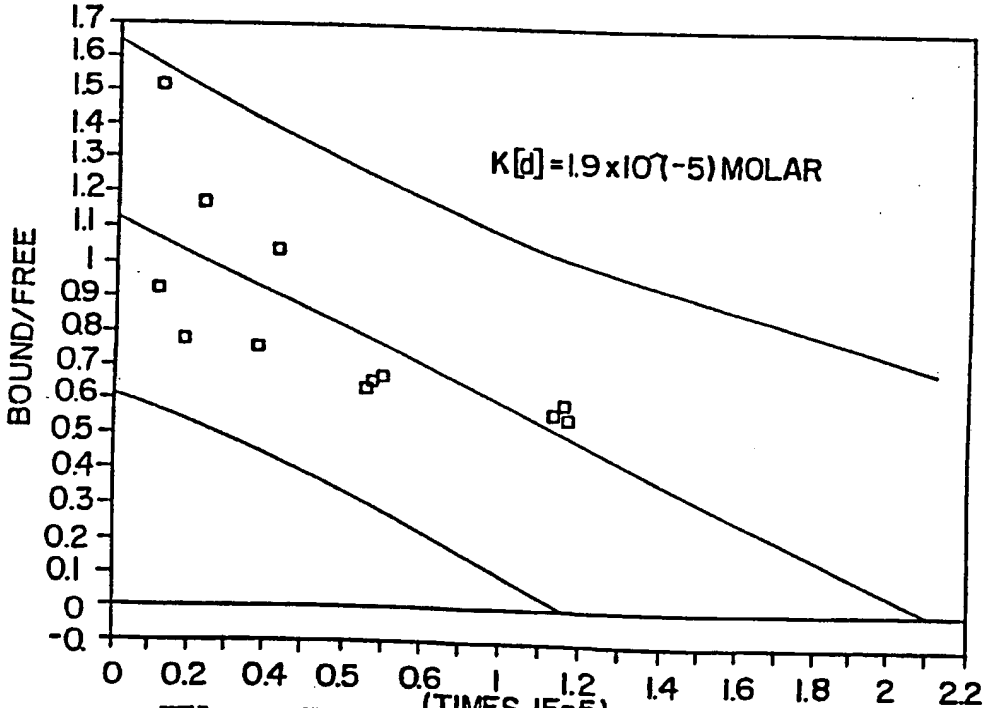
(TIMES 1E-5)
BOUND (MOLAR)

Fig. 4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BOUND (MOLAR)

Fig. 5

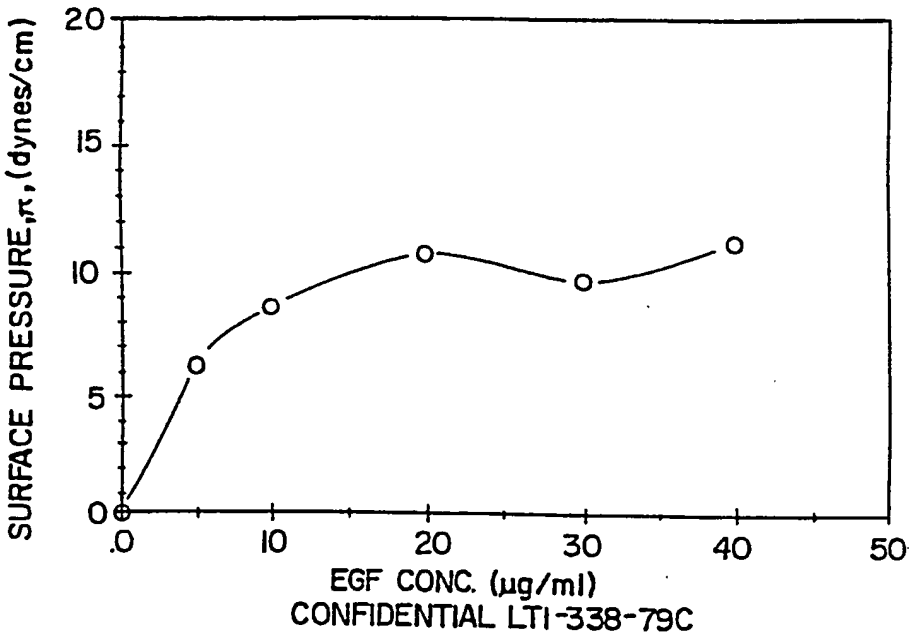


Fig. 6

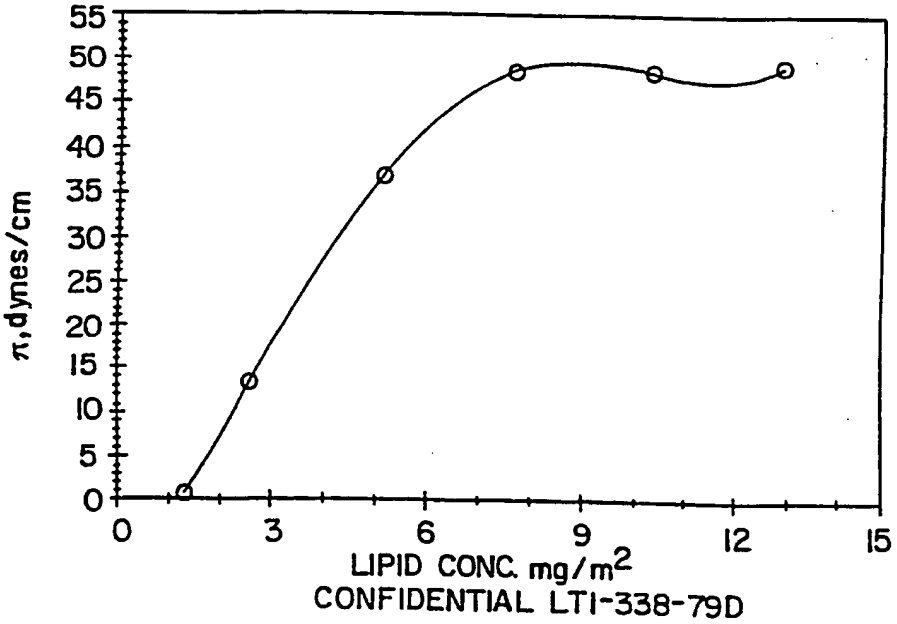
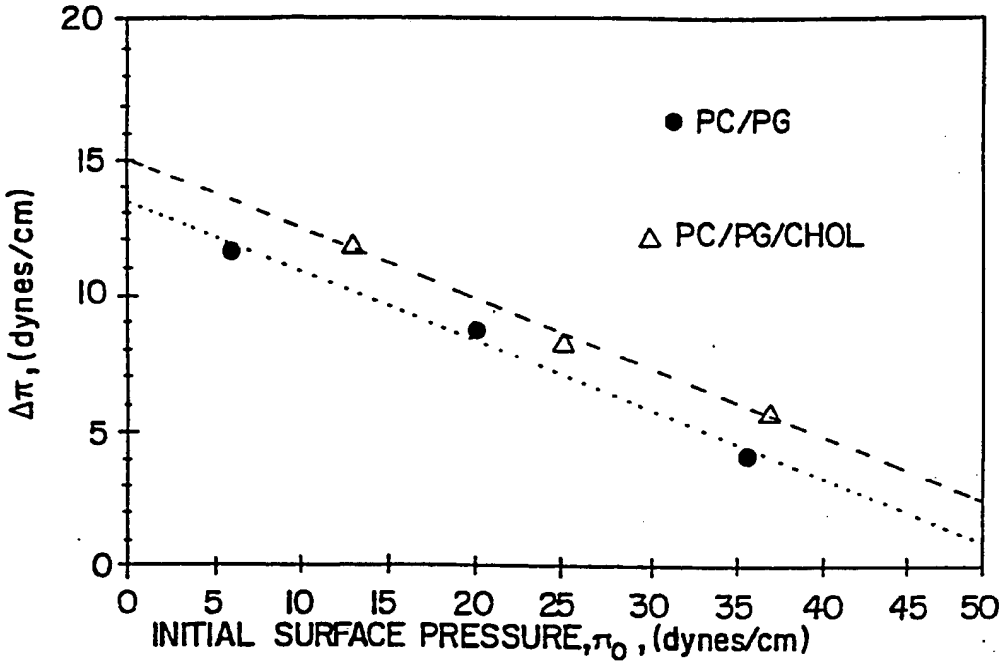


Fig. 7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15
10
5
0
Δπ, (dynes/cm)
● PC/PG
△ PC/PG/CHOL
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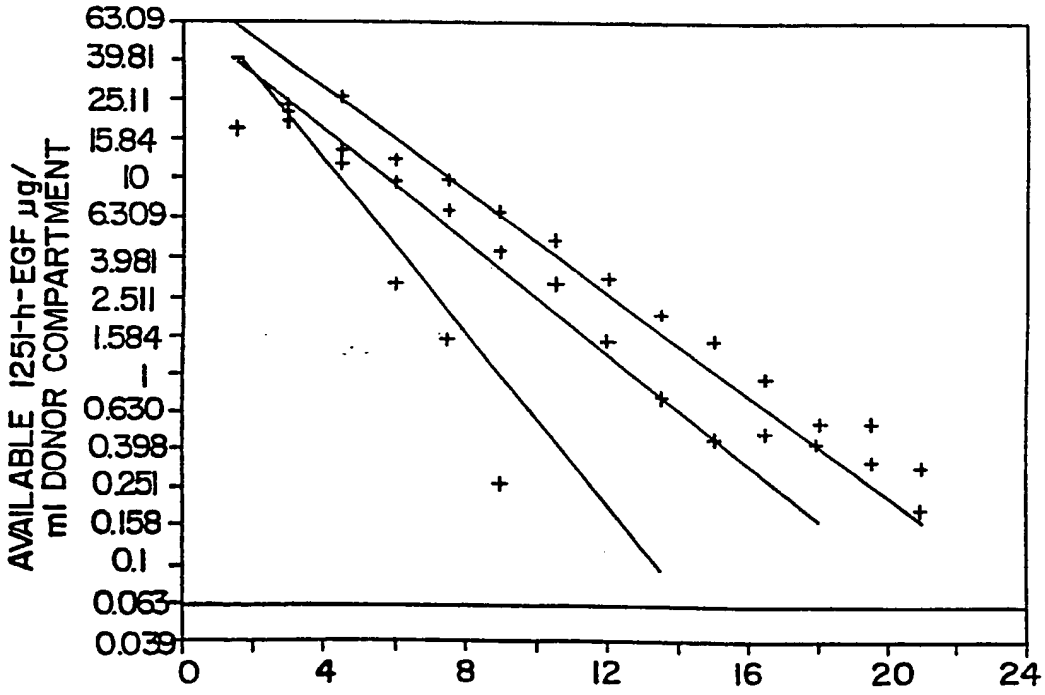
INITIAL SURFACE PRESSURE, π_0, (dynes/cm)

Fig. 8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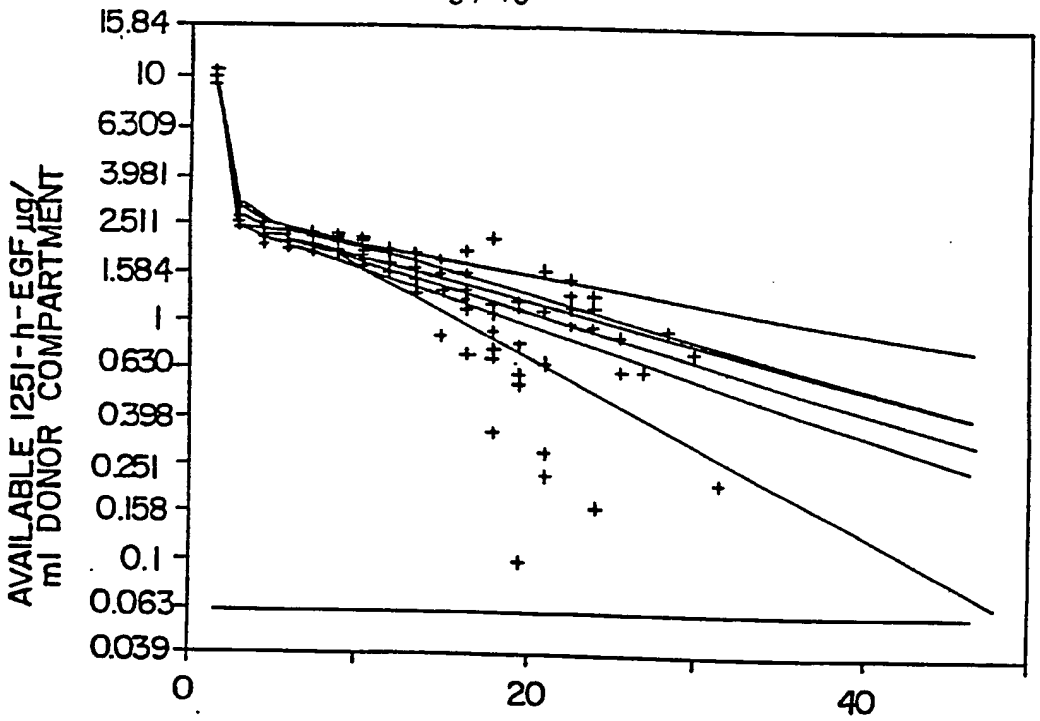
25.11
15.84
10
6.309
3.981
2.511
1.584
1
0.630
0.398
0.251
0.158
0.1
0.063
0.039
AVAILABLE 125I-h-EGF µg/ ml DONOR COMPARTMENT
0 4 8 12 16 20 24

Fig. 9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2.511
1.584
1
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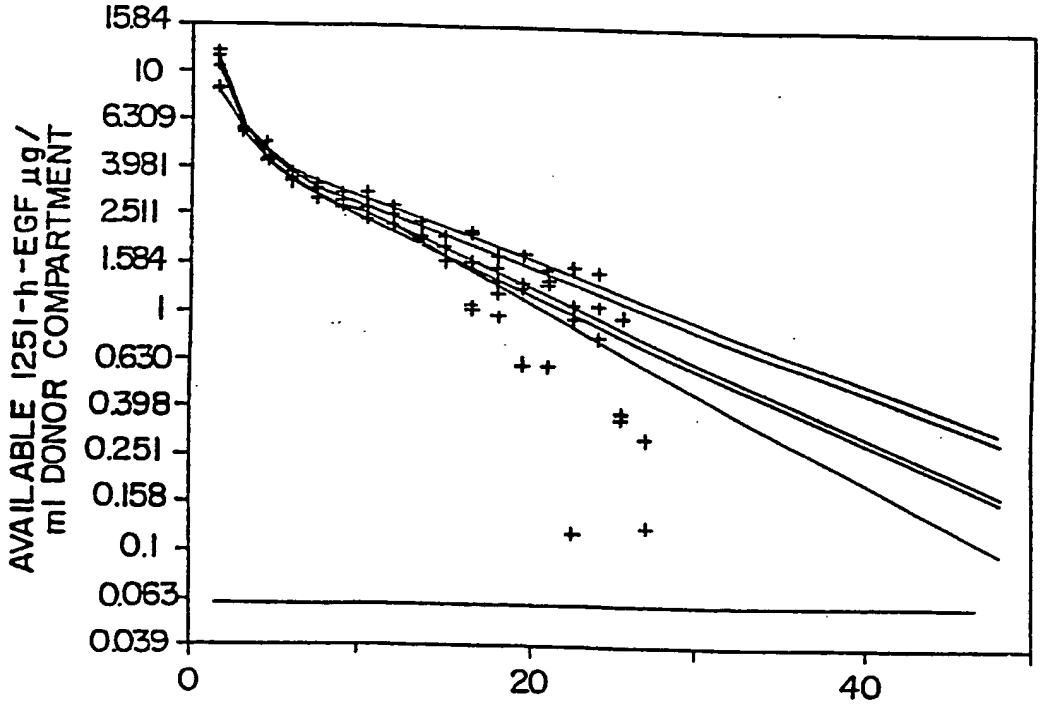
0.398
0.251
0.158
0.1
0.063
0.039
AVAILABLE 125I-h-EGF µg/ ml DONOR COMPARTMENT
0
20
40

Fig. 10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0.630
0.398
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0.063
0.039
AVAILABLE 125I-h-EGF µg/ ml DONOR COMPARTMENT
0
20
40

Fig. 11

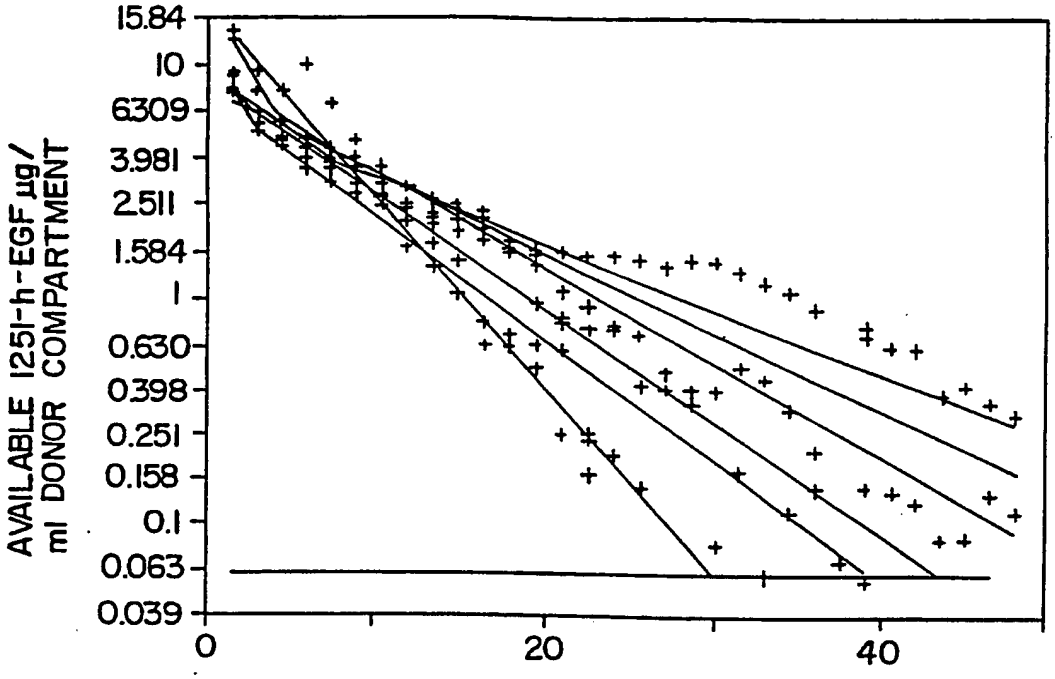


Fig. 12

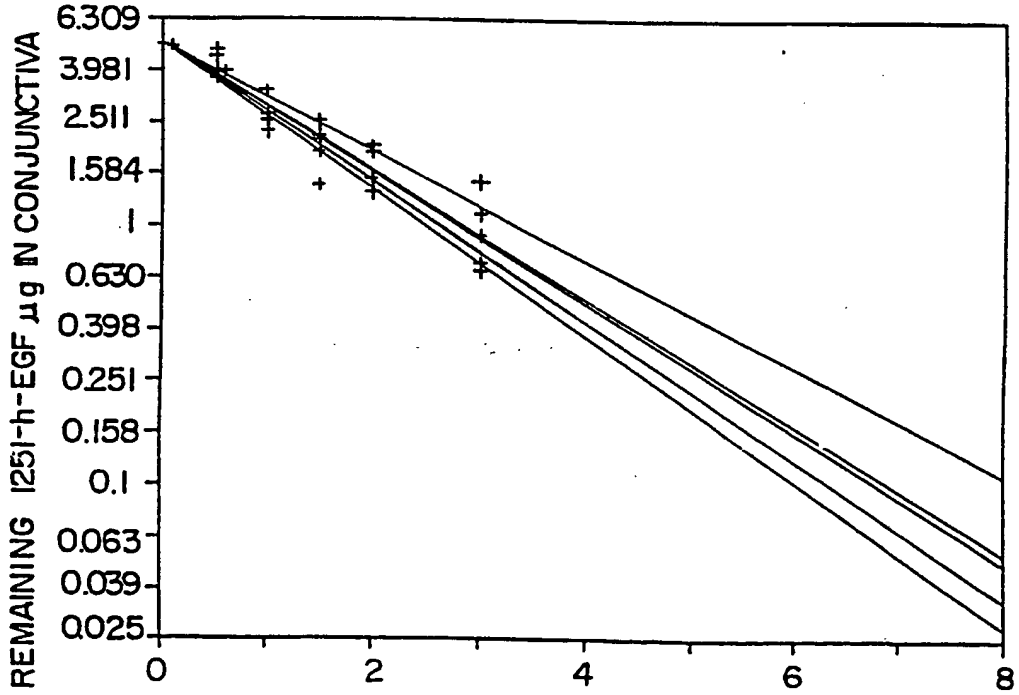
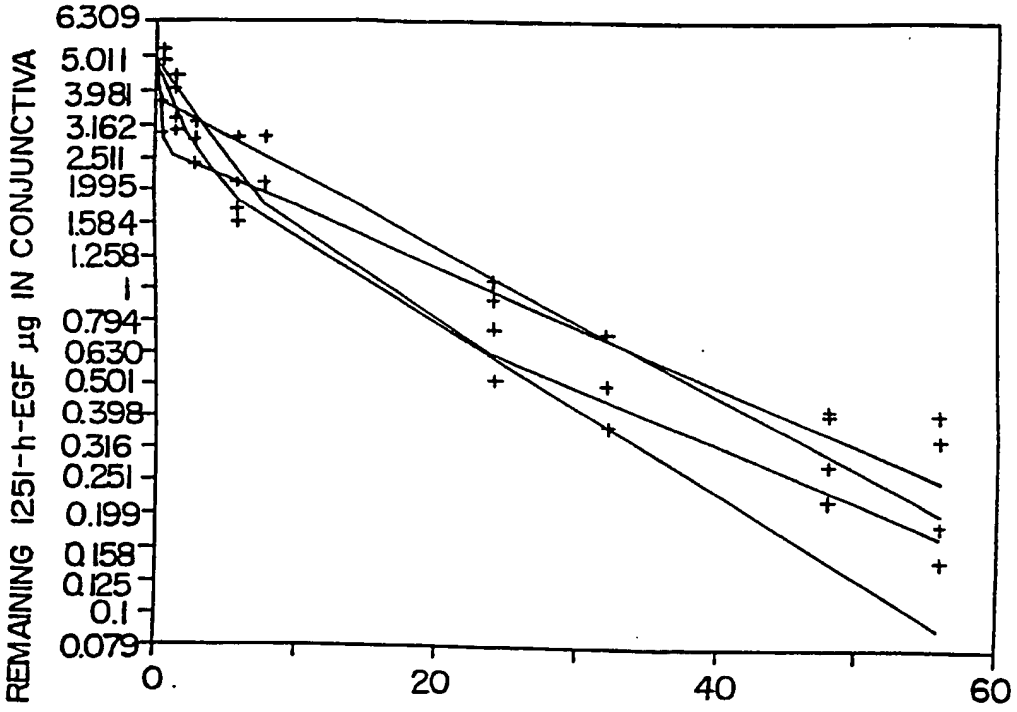


Fig. 13

SHEET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3.162
2.511
1.995
1.584
1.258
1
0.794
0.630
0.501
0.398
0.316
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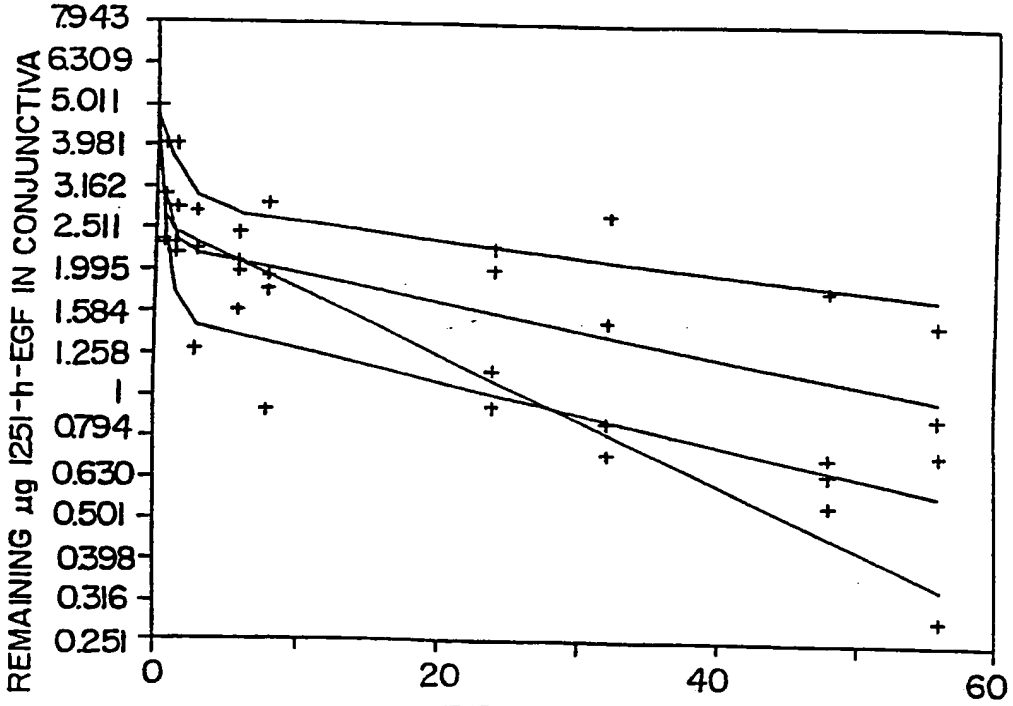
0.158
0.125
0.1
0.079
0
20
40
60

Fig. 14


REMAINING µg 125I-h-EGF IN CONJUNCTIVA
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6.309
5.011
3.981
3.162
2.511
1.995
1.584
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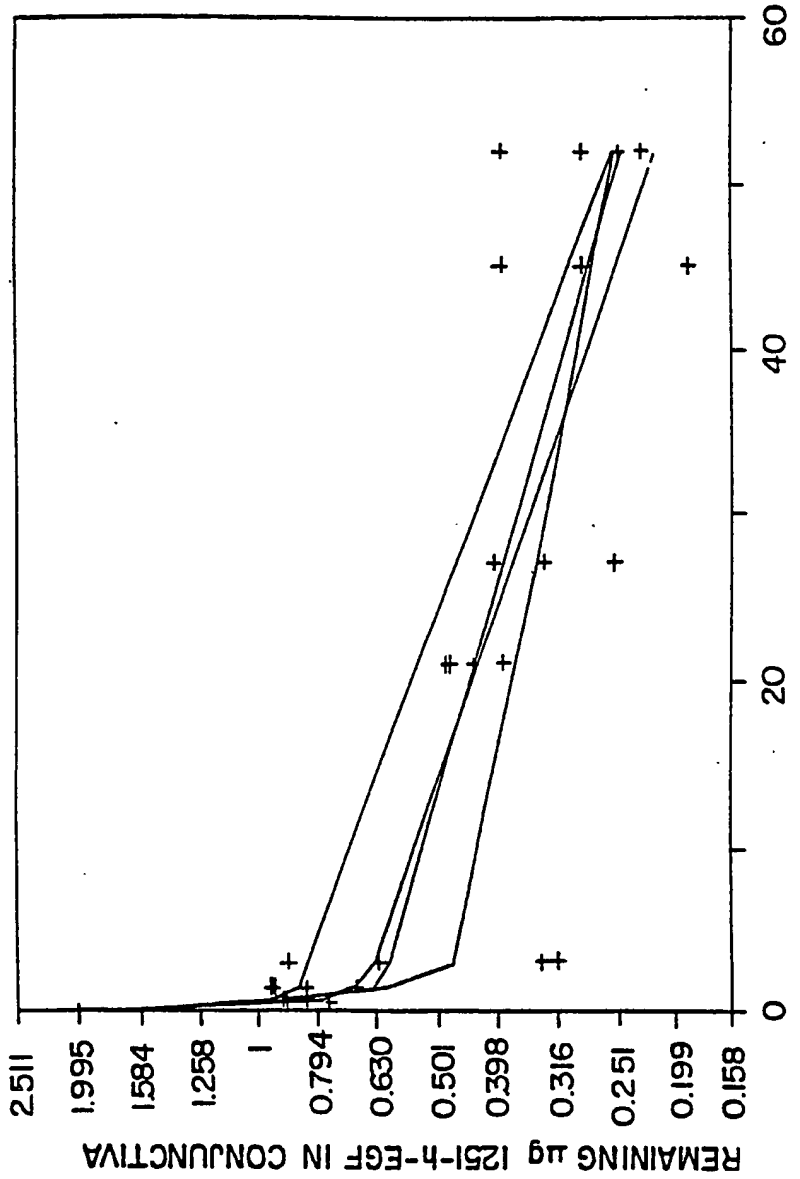
0.251
0
20
40
60

Fig. 15


REMAINING μg 125I-h-EGF IN CONJUNCTIVA
2.511
1.995
1.584
1.258
1
0.794
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0.398
0.316
0.251
0.199
0.158
0
20
40
60

Fig. 16

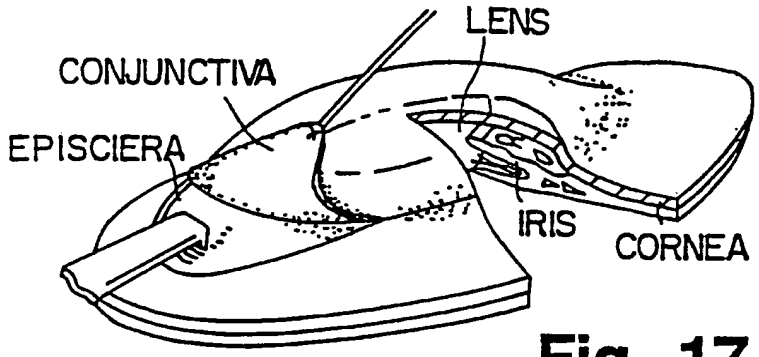

Fig. 17A

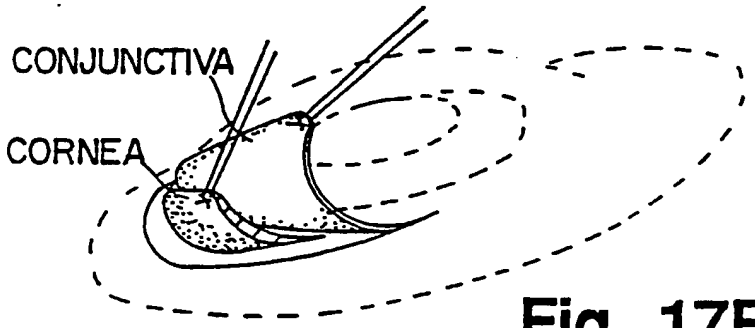

Fig. 17B

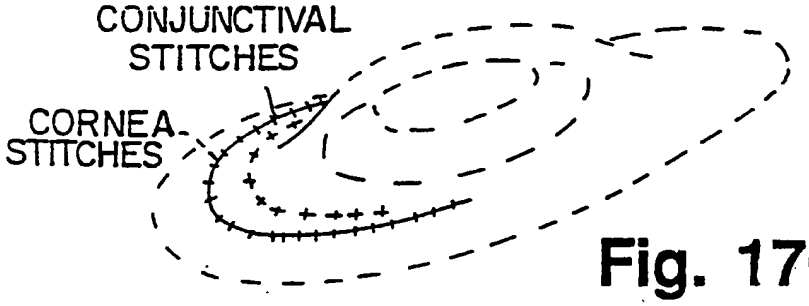

Fig. 17C

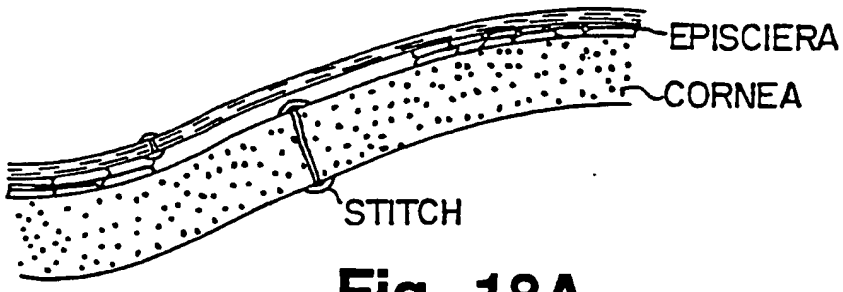

Fig. 18A

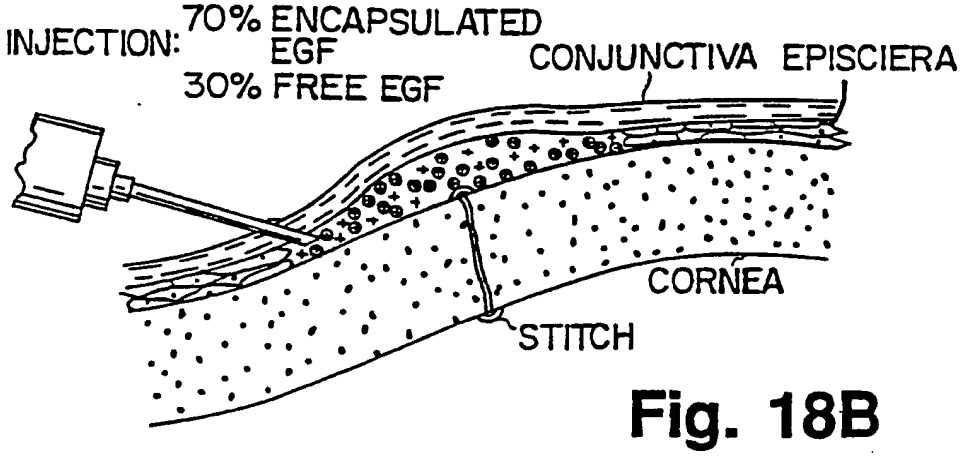

Fig. 18B

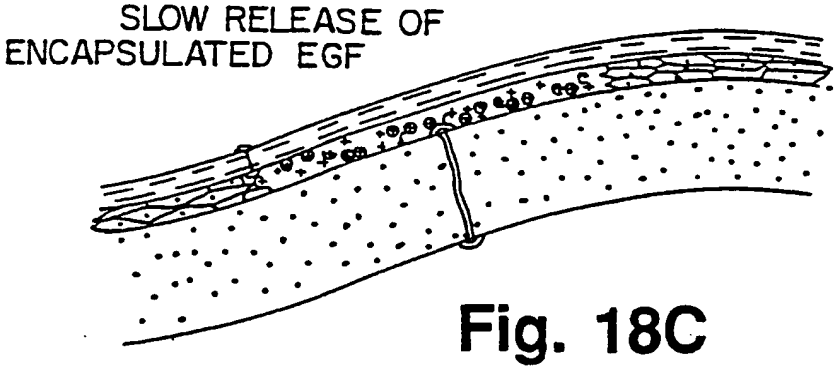

Fig. 18C

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00918

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 9/127, 37/36, 9/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	STN File Server, (karlsruhe, DE), File Medline, (US. Nat. Library of Medicine) G.L. Brown et al.: "Acceleration of tensile strength of incisions treated with EGF and TGF-beta", AN 89061207, volume 89, & Ann Surg, (1988 Dec) 208 (6) 788-94 see the abstract --	1-16
X,Y	WO, A, 88/00824 (LIPOSOME TECHN. INC.) 11 February 1988 see page 6, paragraph 2; page 30, line 11 - page 31, line 4; claim 7; claim 9, line 9 & US, A, 4804539 (cited in the application) --	1-16

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th June 1990	16 JUL 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	STN File Server, (Karlsruhe, DE), File Biosis, volume 89, nr 17568, AN 89:292224,. Y. Ishii et al.: "Preparation of EGF labeled liposomes and their uptake by hepatocytes", pages 732-736 & Biochem Biophys res commun 160 (2). 1989. 732-736. coden: BBRCA9 see the whole abstract	1,17
A	EP, A, 0162724 (VESTAR) 27 November 1985 see the whole document	1-17

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 18-20 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT-Rule 39.1 (iv): methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9000918 SA 35105

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8800824	11-02-88	US-A- 4839175	13-06-89
		US-A- 4804539	14-02-89
		EP-A- 0316345	24-05-89
EP-A- 0162724	27-11-85	AU-B- 576363	25-08-88
		AU-A- 4285685	12-12-85
		CA-A- 1263311	28-11-89
		JP-A- 60258109	20-12-85